CLONING AND CHARACTERISATION OF GONADOTROPIN-RELEASING HORMONE RECEPTORS FROM SPECIES IN NON-MAMMALIAN VERTEBRATE CLASSES: AMPHIBIA AND OSTEICHTHYES

Thesis presented for the Degree of

DOCTOR OF PHILOSOPHY

In the Department of Chemical Pathology

UNIVERSITY OF CAPE TOWN

July 1998

by

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B.Sc (Hons)

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Acknowledgements

I would firstly like to acknowledge my supervisors, Prof Nicola Illing and Prof Robert Millar, for their excellent supervision and their support, both intellectually and financially. I would also like to thank them for their enthusiasim, which makes it worthwhile and also for their friendship. I would also like to thank Dr Judy King for her interest in this work and the help, which she provided in several areas. I would like to thank the Department of Chemical Pathology, University of Cape Town for allowing me the opportunity to complete this work.

I would like to thank Mr J. Morta for technical assistance and Drs RE. Peter (University of Alberta) and Y-Y. Peng (University of Chicago) for supplying goldfish and bullfrog materials

I would also like to acknowledge the support of my husband and family whose encouragement and support I really appreciate.

Abstract

Two or more forms of gonadotropin-releasing hormone (GnRH) have been isolated from most vertebrate species. In most species, GnRH variants have been shown to occur in distinct areas of the peripheral and central nervous systems, the gonads and other peripheral organs. Although GnRH is a primary regulator of gonadotropin secretion, it has been shown to have additional roles such as the regulation of growth hormone secretion in goldfish and the inhibition of a potassium current (M-current) in amphibian sympathetic ganglia. This raises the possibility of the occurrence of multiple GnRH receptor subtypes. This thesis describes the cloning and characterisation of GnRH receptor subtypes from two non-mammalian vertebrates, the Amphibian, *Xenopus laevis* and the Osteichthyes, *Carassius auratus* (goldfish). Using degenerate primers designed to the mammalian GnRH receptors two putative receptor subtypes were identified from both *X. laevis* (*Xl*a.1 and *Xl*b.1) and goldfish (GfA and GfB) genomic DNA. The full-length cDNA for *Xl*a.1, was cloned from pituitary cDNA. When transiently expressed in COS-1 cells, this clone showed a GnRH-dependent stimulation of inositol phosphates. No full-length clone for *Xl*b.1 could be isolated using cDNA from several different tissues. A partially processed transcript was, however, amplified from sympathetic ganglia cDNA. These ganglia showed specific binding to a chicken GnRH II (cGnRH II) agonist and cGnRH II immunoreactivity was also detected in extracts from the ganglia. The expression, function and pharmacology of clone *Xl*b.1, thus remains unknown, but the presence of cGnRH II-specific binding sites on membranes from the sympathetic ganglia with distinctly different pharamcology, implies the presence of a second GnRH receptor subtype in these neurons. Full-length cDNA clones of GfA and GfB were amplified from goldfish pituitary and brain cDNA respectively. These receptors had a 71% amino acid identity to each other and a 43% amino acid identity to the human GnRH receptor. The pharmacology of these two GnRH receptor subtypes was investigated by transient expression in COS-1 cells. The GfA and GfB receptors had different pharmacologies as demonstrated by their selectivities for GnRH analogues. *In situ* hybridisation revealed a distinct expression pattern of the goldfish GnRH receptor subtypes in the brain, gonads and liver (Dr R. Peter, University of Alberta). The full-length receptors cloned from the pituitaries and brain of *X. laevis* and the goldfish have a low homology to the cloned mammalian GnRH receptors and have several different features, such as the presence of an intracellular carboxy-terminal tail. This thesis, describing the primary structure and characterisation of ligand selectivity of non-mammalian GnRH receptors, provides some useful foundations for future work towards understanding ligand recognition in the GnRH receptor. The description of multiple receptor subtypes in the goldfish and possibly in *X. laevis* also provides valuable information into alternative roles of GnRH and its receptor, which we are only beginning to understand.

List of Abbreviations

bp	Base pairs
cAMP	Cyclic adenosine monophosphate
cDNA	Complimentory DNA
cGnRH I	Chicken GnRH I ([Gln^8]GnRH)
cGnRH II	Chicken GnRH II ([His^5,Trp^7,Tyr^8]GnRH)
cpm	Counts per minute
DAG	Diacylglycerol
ED_{50}	Peptide concentration required to half maximally stimulate inositol phosphate production
EL	Extracellular loop
FSH	Follicle stimulating hormone
GABA	Gamma-aminobutyric acid
GAP	GnRH-associated protein
GH	Growth hormone
GnRH	Gonadotropin-releasing hormone
GPCR	G-protein coupled receptor
GtH	Gonadotropin
GTP	Guanine triphosphate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPLC	High performance liquid chromatography
IC_{50}	Peptide concentration required to half maximally inhibit binding of labelled GnRH peptides
IL	Intracellular loop
IP	Inositol Phosphate
IP_3	1,4,5-trisphosphate
kbp	Kilo base pairs
LH	Luteinsing hormone
M	Molarity
M-current	Muscarinic-current
mGnRH	Mammalian GnRH
mRNA	Messenger RNA
NE	Norepinephrine
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NPY	Neuropeptide Y
pGlu	Pyro-glutamate
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
RIA	Radioimmunoassay
sbGnRH	Seabream GnRH, [Ser^8]GnRH
sGnRH	Salmon GnRH, [Trp^7,Leu^8]GnRH
Ta	Annealing temperature
TM	Transmembrane domain
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol

Introduction

1.1 Summary

Gonadotropin-releasing hormone (GnRH) is a decapeptide, which is synthesised in neurons of the hypothalamus. GnRH is secreted in a pulsatile manner directly into the hypophysioportal system. GnRH binds to a specific receptor on the surface of gonadotropes in the anterior pituitary, where it acts as a central regulator of reproductive function by stimulating the release of the heterodimeric gonadotropins luteinising hormone (LH) and follicle stimulating hormone (FSH). These hormones stimulate gonadal production of sex steroids resulting in gametogenesis. Twelve different forms of GnRH have been identified to date, and they are named from the species in which they were first identified. In most of the vertebrate species analysed to date two or more forms of GnRH occur. The highly conserved and widely distributed [His^5,Trp^7,Tyr^8]GnRH (chicken GnRH II; cGnRH II) usually co-occurs with one or more variable forms of GnRH. The physiological relevance of the occurrence of more than one form of GnRH in a single species is unknown, but implies a more widespread function, which correlates to its widespread distribution even in non-reproductive tissues. GnRH has been shown to be important in reproductive behaviour and neuromodulation or neurotransmission. The GnRH receptors from several mammalian and one non-mammalian species have been cloned. The mammalian GnRH receptors, which are highly conserved have been shown to be more selective for mammalian GnRH (mGnRH), than the other endogenous forms of GnRH. These receptors are G-Protein coupled receptors (GPCRs) which couple to the $G_{q/11}$ class of G-Proteins.

1.2 Introduction

This introduction aims to give a brief background of the structures, evolution, function and regulation of gonadotropin-releasing hormone (GnRH) in vertebrates. The structure/activity relations and biologically relevant conformations will be discussed. Finally the GnRH receptor structure, function, signal propagation and termination, and receptor expression will be described for the mammalian system.

1.3 The Primary Structure of GnRH

Gonadotropin-releasing hormone (GnRH) is a decapeptide, which regulates the synthesis and release of luteinising hormone (LH) and follicle stimulating hormone (FSH) [for reviews, see Schally *et al.*, 1971; Kiesel, 1993]. GnRH was originally isolated from mammalian hypothalami [Matsuo *et al.*, 1971; Burgus *et al.*, 1972], but structural variants have been identified in a range of vertebrates, including the primitive protochordates. Twelve forms of the peptide have been isolated to date, and named from the species in which they were first identified [Gothilf *et al.*, 1995; Powell *et al.*, 1996; Jimenez-Liñan *et al.*, 1997, for reviews, see Sherwood *et al.*, 1993; 1997; King and Millar 1995; 1997].

	1	2	3	4	5	6	7	8	9	10
Mammal	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly.NH_2
Chicken I	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Gln	Pro	Gly.NH_2
Seabream	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Ser	Pro	Gly.NH_2
Catfish	pGlu	His	Trp	Ser	His	Gly	Leu	Asn	Pro	Gly.NH_2
Salmon	pGlu	His	Trp	Ser	Tyr	Gly	Trp	Leu	Pro	Gly.NH_2
Dogfish	pGlu	His	Trp	Ser	His	Gly	Trp	Leu	Pro	Gly.NH_2
Chicken II	pGlu	His	Trp	Ser	His	Gly	Trp	Tyr	Pro	Gly.NH_2
Guinea Pig	pGlu	**Tyr**	Trp	Ser	Tyr	Gly	Val	Arg	Pro	Gly.NH_2
Lamprey III	pGlu	His	Trp	Ser	His	Asp	Trp	Lys	Pro	Gly.NH_2
Lamprey I	pGlu	His	**Tyr**	Ser	Leu	Glu	Trp	Lys	Pro	Gly.NH_2
Tunicate I	pGlu	His	Trp	Ser	Asp	Tyr	Phe	Lys	Pro	Gly.NH_2
Tunicate II	pGlu	His	Trp	Ser	Leu	Cys	His	Ala	Pro	Gly.NH_2

Figure 1.1. Primary structures of the twelve known forms of GnRH isolated from vertebrate and protochordate brains. Shaded boxes show highly conserved regions.

The primary structure of the different forms of GnRH reveals highly conserved NH_2 and COOH terminal sequences, with a variable domain from residues 5 to 8 (fig. 1.1). Position 8 is the most variable followed by positions 5, 7 and 6. Further structural variation in the peptide may result from post-translational modifications. The hydroxylation of the proline in position 9 of mammalian GnRH (mGnRH) has been demonstrated in rat and frog brains [Gautron *et al.*, 1991] and proteolytic cleavage may create novel activities [Moss *et al.*, 1973].

In all species analysed to date, GnRH is encoded by a gene containing four exons. GnRH is synthesised as a precursor hormone, which is enzymatically processed to form the secreted decapeptide. The precursors of mGnRH [Seeburg and Adelman, 1984; Adelman *et al.*, 1986; Hayes *et al.*, 1994] chicken GnRH I [Dunn *et al.*, 1993], salmon GnRH [Bond *et al.*, 1991; Suzuki *et al.*, 1992; Klungland *et al.*, 1992; Gothilf *et al.*, 1995; Okuzawa *et al.*, 1994], cGnRH II [Bogerd *et al.*, 1994; Gothilf *et al.*, 1995; White *et al.*, 1994], catfish GnRH [Bogerd *et al.*, 1994], and seabream GnRH [Gothilf *et al.*, 1995; White *et al.*, 1995] have been studied in several species of vertebrates [for review, see King and Millar, 1997]. The mammalian hormone is synthesised in the hypothalamus as a preprohormone of 92 amino acids [Seeburg and Adelman, 1984; Adelman *et al.*, 1986]. This precursor consists of a 21-23 amino acid signal sequence, followed by the decapeptide and a Gly-Lys-Arg sequence, which is required for enzymatic processing and amidation of Gly^{10}. The amino-terminal pyro-glutamate forms by a spontaneous cyclisation of Gln^{1}. The remaining amino acids form the GnRH-associated peptide. The role of this peptide is unknown, but it was postulated to inhibit prolactin secretion [Nikolics *et al.*, 1985] and increase gonadotropin secretion [Millar *et al.*, 1986(a); Milton *et al.*, 1986]. This peptide is, however, quite conserved, even amongst the different forms of GnRH, therefore, inferring the possibility of a physiological role [for review, see King and Millar, 1995; 1997].

1.4 The Evolution of GnRH Structural Variants

Although the primary structure of GnRH has changed over more than 500 million years of evolution, it remains pivotal in controlling reproduction from protochordates, to higher vertebrates. The multiple forms of GnRH appear to have arisen by an early gene duplication of an ancestral GnRH gene [for reviews, see King and Millar, 1995;

1997; Sherwood *et al.*, 1993; 1997]. This ancestral molecule may have evolved to form the conserved cGnRH II on the one arm, while a further duplication, gave rise to the other GnRH isoforms (fig. 1.2). A recent phylogenetic analysis, predicts the existence of three evolutionarily distinct forms of GnRH as characterised in vertebrates by their brain distribution [White *et al.*, 1998]. These include those localised in the hypothalamic area (GnRH I), the mesencephalic area (GnRH II) and the telencephalic area (GnRH III). GnRH I functions mainly as a hypophysiotropic factor, and includes the mGnRH, Seabream GnRH (sbGnRH) and chicken GnRH I (cGnRH I) subtypes. The highly conserved GnRH II (cGnRH II) has an unknown function, but may act as a neuromodulator [Jan and Jan, 1980]. The GnRH III subtype including the salmon GnRH isoform, which has been isolated in bony fish, may represent a class of GnRH only recently identified in mammals [Montaner *et al.*, 1997]. These evolutionary schemes [White *et al.*, 1998; King and Millar, 1997; Sherwood *et al.*, 1997], all support the hypothesis of an early duplication of an ancestral gene before species divergence.

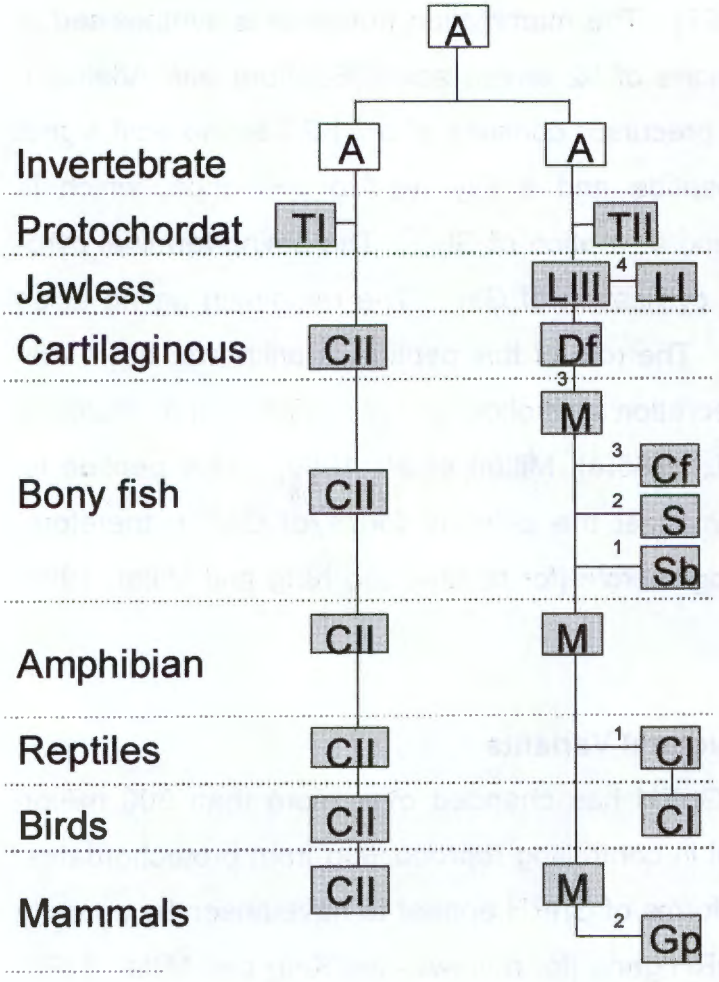


Figure 1.2. King and Millar (1995; 1997) proposed a possible evolutionary scheme for the GnRH gene. The scheme involves the early duplication of an ancestral gene (A), which may have led to the other GnRH forms. TI, Tunicate GnRH I; TII, Tunicate GnRH II; LIII, Lamprey GnRH III; LI, Lamprey GnRH I; Df, Dogfish GnRH; M, mammalian GnRH; Cf, catfish GnRH; S, salmon GnRH; Sb, Seabream GnRH; CI, chicken I; Gp, guinea pig GnRH and CII, chicken GnRH II.

been seen in the common carp, *Cyprinus carpio* [Lin *et al.*, 1993] and *Tilapia* hybrids [Melamed *et al.*, 1995]. There is also some evidence, although unconfirmed for GnRH-stimulated GH secretion in the rainbow trout, *Oncorhynchus mykiss* [Blaise *et al.*, 1995; for review, see LeGac *et al.*, 1993]. In the African catfish, however, GnRH does not affect GH secretion [Bosma *et al.*, 1997]. GnRH has also been shown to stimulate pituitary GH secretion in rat neonates [Andries and Denef, 1995]. The occurrence of salmon GnRH in the neurons of the olfactory bulb, with projections into the retina of the African catfish suggest that this peptide may also co-ordinate sensory input with reproductive behaviour [Van Weerd *et al.*, 1991]. GnRH also plays an important role as a paracrine/autocrine regulator in the gonads. Transcription of the GnRH gene has been described in the ovaries [Oikawa *et al.*, 1990; Goubau *et al.*, 1992], where it enhances the basal production of steroids, but attenuates the gonadotropin-induced cAMP steroidogenesis. This may occur, either, by increased degradation or decreased production of cAMP [Harwood *et al.*, 1980; Knecht and Catt, 1981, Ranta *et al.*, 1983]. The occurrence of GnRH and GnRH receptors in non-reproductive tissue, such as liver, heart kidney and skeletal muscle by Kakar and Jennes (1995) by reverse-transcriptase PCR, may implicate GnRH involvement in the regulation of cellular functions unrelated, or related to reproduction. GnRH has also been shown to have an autocrine role in certain tumours, indicated by the presence of specific GnRH binding sites on prostrate tumours [Hierowski *et al.*, 1983], in breast carcinoma cell lines, which express mRNA of both GnRH and its receptor [Eidne *et al.*, 1985; Eidne *et al.*, 1987; Harris *et al.*, 1988; Harris *et al.*, 1991] and in tumours originating from the endometrium and ovary [Imai *et al.*, 1994(a); 1994(b)].

1.6 GnRH regulation

Tissue specific regulation of GnRH in mammals is currently an active area of research. The 5' flanking sequences of the rat [Kepa *et al.*, 1992; Whyte *et al.*, 1995] and human [Dong *et al.*, 1993, 1997] GnRH genes have been well characterised. Both upstream sequences appear to contain several putative transcription factor recognition sequences, and both the rat and the human sequences appear to have neuron-specific recognition sequences, which infer tissue specificity. Interestingly the human promoter appears to have two independent promoter regions. Reproductive tissues for example the gonads, use a more 5' upstream promoter and trancription

1.5 The role of GnRH

The physiological significance of the occurrence of multiple forms of GnRH within a single species is unclear. The hypothalamic form of GnRH is important for the regulation of pituitary gonadotropin release. The mesencephalic form of GnRH, namely, cGnRH II (GnRH II) has been highly conserved throughout vertebrate evolution. cGnRH II was originally not found in advanced eutherian species, but has recently been described in the brain of primitive placental mammals such as, the musk shrew [Dellovade *et al.*, 1993; King *et al.*, 1994(b)], and the tree shrew [Kasten *et al.*, 1996] and more recently in the brain of primates [Lescheid *et al.*, 1997] and human brain and kidneys [White *et al.*, 1998]. The role of this highly conserved form of GnRH, which predominates extra-hypothalamically is unknown. Although able to stimulate the release of gonadotropins, in some cases more potently than the hypothalamic GnRH I form [for reviews, see King and Millar, 1995; Sealfon *et al.*, 1997], it is thought that it may have an additional role in neuromodulation or neurotransmission. In amphibia, cGnRH II has been shown to potently inhibit the modulatory late-slow postsynaptic potential, known as M-current [Jan and Jan, 1980; 1982; Jones 1987]. It has also been implicated in the control of reproductive motor behaviour [Miller and Kriebel, 1986; Maney *et al.*, 1997; for review, see Rissman *et al.*, 1997]. The high conservation and widespread distribution of cGnRH II may imply selective pressure from multiple functions, related to reproductive behaviour and functioning.

Behavioural interactions have also been shown to be important in the regulation of hypothalamic GnRH neuron size or number. Such interactions have been shown to alter LH-release [Bowman *et al.*, 1987; Kaynard *et al.*, 1990], which may be a reflection of the increase in GnRH immunoreactive neurons, observed after a behavioural stimulus [for review, see Rissman *et al.*, 1997]. This increase in GnRH neurons may be linked to steroid hormone level changes. An example of this is the inverse ratio between GnRH neuronal size and cortisol levels, which is seen in the territorial African cichlid fish, *Haplochromis burtoni* [Fox *et al.*, 1997; Fernald, 1997].

GnRH can also stimulate the release of growth hormone in goldfish, *Carassius auratus* [Marchant *et al.*, 1989] apparently through the stimulation of a different GnRH receptor subtype [Murthy and Peter, 1994]. This additional role of GnRH has also

start site than do hypothalamic tissues, resulting in a smaller mRNA transcript in hypothalamic tissues [Dong *et al.*, 1997]. This may, therefore, be an important mechanism for differential control and tissue specificity. The promoter regions also have an AP-1 site, which is a known site for the interaction of transcription factors such as c-*fos*. Several growth factors and tissue-specific enhancers may be involved in the regulation of the GnRH gene. For example, insulin-like growth factor I has been shown to increase both human and rodent GnRH gene expression via the AP-1 site [Zhen *et al.*, 1997].

In mammals, GnRH is released from the median eminence of the hypothalamus into the portal system, in a pulsatile manner. This pulsatile release is required to maintain gonadotropin secretion [for review, see Conn and Crowley, 1991]. The amplitude and frequency of these pulses vary with reproductive states, for example the frequency increases during the follicular phase and slows during the luteal phase of the human menstrual cycle [Reame *et al.*, 1984]. There is also evidence to suggest that the frequency and amplitude of the GnRH pulses can regulate gonadotropin subunit gene expression [Dalkin *et al.*, 1989], possibly by increasing gene transcription [Haisenleder *et al.*, 1991; 1993]. The regulation may also involve GnRH receptor number [Kaiser *et al.*, 1997]. The pulsatile release of GnRH from stores in the hypothalamus may be under the control of several inhibitory and excitatory neurotransmitters. A putative model for the interaction of these neurotransmitters has been proposed by Brann and Mahesh [1997] (fig. 1.3). The neurotransmitters are regulated by the steroid hormones, estradiol and progesterone. The major inhibitory neurotransmitters are the endogenous opioid peptides, for example, β-endorphin, and neuropeptide K. The opioid peptides, which are activated by the cytokine, interleukin-1β [Kalra *et al.*, 1990], have been shown to inhibit luteinising hormone secretion [Rivier and Vale, 1990]. The tachykinin, neuropeptide K-containing neurons, which have been immunocytochemically co-localised with the hyphothalamic GnRH neurons [Valentino *et al.*, 1986], have also been shown to have an inhibitory effect on luteinising hormone secretion [Sahu and Kalra, 1992]. These neurotransmitter systems act through the inhibition of the excitatory amino acid glutamate [Bonavera *et al.*, 1993]. Gamma-aminobutyric acid (GABA), which is secreted by neurons in the hypothalamic area, is released episodically into the medial pre-optic area in synchrony with luteinising hormone pulses. GABA is thought

to restrain amplitude and duration of GnRH secretion. Glutamate along with nitric oxide (NO) [Bhat *et al.*, 1995], neuropeptide Y (NPY), nor-epinephrine (NE) and galanin are thought to act alone or together to stimulate GnRH secretion [for review, see Kalra *et al.*, 1997].

In summary then, during non-surge conditions the inhibitors act by attenuating the effects of the stimulatory neurotransmitters. During surge conditions rising levels of steroid hormones are thought to inhibit opioid neurons thereby allowing the activation of the excitatory components [for review, see Brann and Mahesh, 1997].

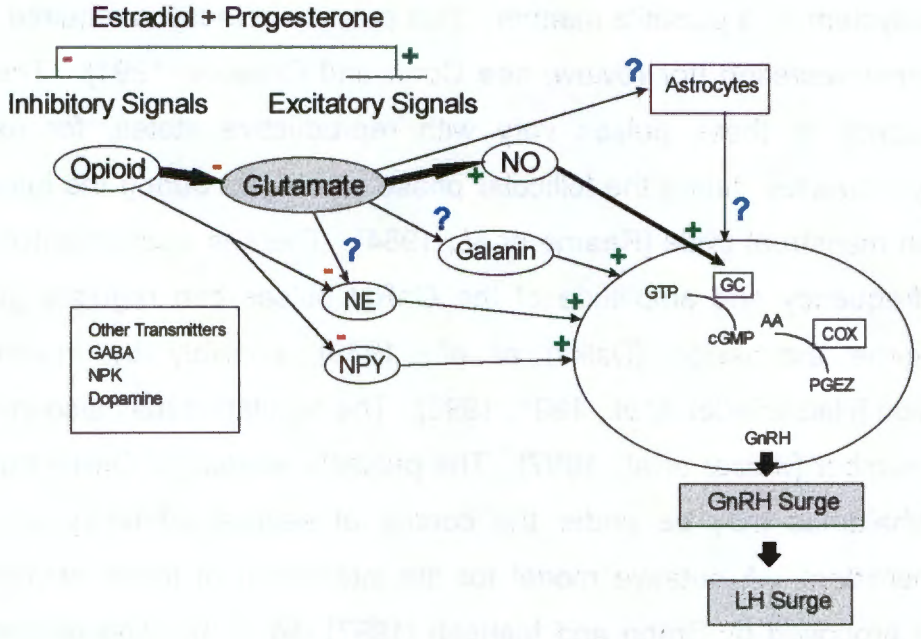


Figure 1.3. A proposed model for the pulsatile control of GnRH secretion [Brann and Mahesh, 1997]

1.7 GnRH as a gonadotropin-releasing factor

The main target of GnRH is the anterior pituitary where it binds to GnRH receptors on the surface of gonadotropes. Once bound, the receptor, which is a G-Protein coupled receptor, is activated. The activated receptor in turn activates the $G_{q/11}$ class of heterotrimeric GTP-binding proteins, which results in a cascade of intracellular signaling events, ultimately leading to the synthesis and release of LH and FSH. The activation of the G-Protein by the receptor is catalytic; thus several G-proteins can be activated by one receptor, thereby, amplifying the signal.

The intracellular signaling events leading to the release of the gonadotropins are summarised in fig. 1.4 [for reviews, see Kiesel, 1993; Stojilkovic *et al.*, 1994]. Briefly, ligand binding to the GnRH receptor activates the G-protein, causing the α, β, and γ subunits to dissociate and form the G_{α}-GTP and the $G_{\beta\gamma}$ subunits. Both the G_{α} and $G_{\beta\gamma}$ subunits are capable of regulating intracellular signaling. GTP is hydrolysed to GDP by the GTPase activity of the G_{α} subunit causing reassociation with the $G\beta\gamma$-subunits and subsequent inactivation of the G-protein [for review, see Van Biesen *et al.*, 1996]. Activation of the $G_{q/11}$ class of G-proteins, in the gonadotrope cells in the anterior pituitary activates the phosphatidylinositol cycle. Phospholipase $C_{\beta 1}$ (PLC), which is activated by the G_{α}- subunit to cleave phosphatidylinositol 4,5-bisphosphate (PIP_2) to form inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) [for review, see Exton, 1994]. Both of these products as well as calcium act as the most important second messengers in the GnRH response. IP_3 stimulates the release of intracellular calcium from vesicular stores, by binding to its receptor. Two calcium responses can be induced by GnRH; a calcium spike/plateau or calcium oscillations. The calcium spike is thought to originate from the release of intracellular calcium, while the plateau phase may be a result of the influx of extracellular calcium. Low concentrations of GnRH seem to induce calcium oscillations, which elevate GnRH receptor numbers and enhance LH gene expression, without stimulating LH release. High concentrations of GnRH cause a calcium spike/plateau stimulus with hormone release, but a decrease in gonadotropin gene expression [Leong and Thorner, 1991]. DAG in combination with calcium and phosphatidylserine, activates protein kinase C (PKC) which phosphorylates serine and threonine residues on target proteins to facilitate gonadotropin release. DAG has also been proposed to be important in feedback control mechanisms, possibly contributing to the calcium oscillatory signal by attenuating PLC activity [Nishizuka, 1986]. PLC may also stimulate GTPase activity of $G_{q/11}$ [Berstein *et al.*, 1992]. DAG also acts as a substrate of phospholipase A_2 (PLA_2), which synthesises arachidonic acid. PLA_2 may be indirectly activated by PKC. Metabolites of arachidonic acid, for example leukotrienes, may be important in gonadotropin modulation [Kiesel *et al.*, 1991]. The GnRH-stimulated release of gonadotropins is, therefore, a highly complex system, finely tuned by the interaction of a number of second messengers.

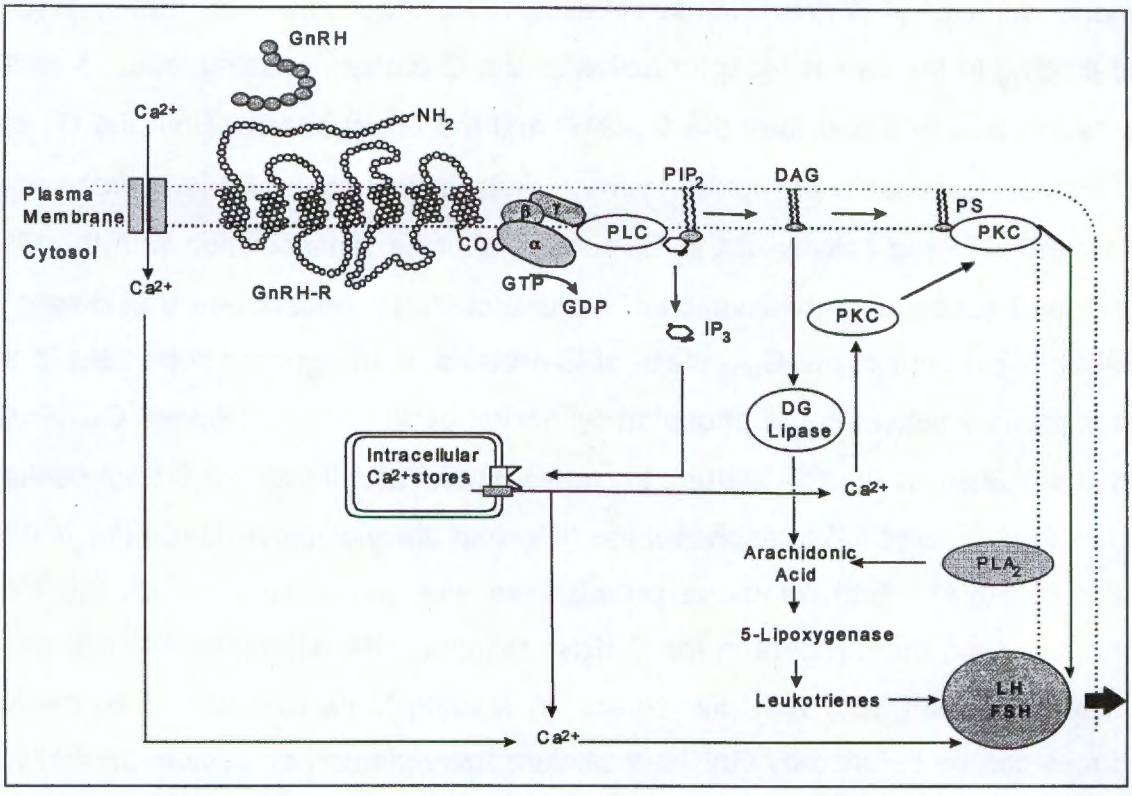


Figure 1.4. Diagram depicting the intracellular signaling events leading to the release of the gonadotropins, LH and FSH, from the anterior pituitary [Kiesel, 1993]. DAG, Diacylglycerol; IP_3, Inositol 1,4,5-triphosphate; PIP_2, Phosphoinositol 4,5-bisphosphate; PS, phosphatidylserine; PLC, Phospholipase C; PKC, Protein Kinase C; PLA_2, Phospholipase A_2.

1.8 Structure/Activity Relations in GnRH

1.8.1 The conformation of GnRH

Studies using nuclear magnetic resonance (NMR) [Deslauriers *et al.*, 1975; Chary *et al.*, 1986] and circular dichroism [Cann *et al.*, 1979], revealed that GnRH is a highly flexible molecule in solution and may exist as a mixture of structural conformations. In order to have a high affinity for the receptor, the most abundant form of the peptide in solution should be the biologically active conformation [Morrison *et al.*, 1987]. The abundant conformation should have the lowest free energy. Momany [1976(a); 1976(b); 1978], used computational analyses of the free energy to predict the possible conformation of GnRH. Despite significant methodological limitations these

studies predicted a β-bend involving amino acids 5 to 8 of GnRH [for review, see Sealfon *et al.*, 1997].

The design of GnRH analogues with conformational constraints, which may stabilise the active conformation have been valuable in determining the biological active conformation [Freidinger *et al.*, 1980]. When the type-II β-turn proposed by Momany was stabilised by the incorporation of a γ-lactam ring between Gly^6 and Leu^7 of mGnRH, the analogue became much more potent. This confirms earlier studies where D-Ala^6, in place of the achiral Gly^6 or L-Ala^6, was presumed to increase the activity of analogues by stabilising the β-II type turn [Monahan *et al.*, 1973].

Two fields of research have contributed to our understanding of the structure and the activity of GnRH. Firstly the study of the activity of naturally occurring GnRHs and secondly the study of synthetic analogues.

1.8.2 Activities of endogenous GnRHs

All the GnRHs stimulate the release of gonadotropins, but the potencies differ considerably, in different vertebrate species (fig. 1.5). Non-mammalian GnRHs, with the exception of cGnRH II have been shown to have poor gonadotropin-releasing activity in mammals [Millar *et al.*, 1986(b); 1989]. The mammalian GnRH receptor, thus, seems to be highly selective for its endogenous ligand, mGnRH. The non-mammalian GnRH receptors were, however, thought to be less selective for the different forms of GnRH, thus binding the structural variants equally [for reviews, see Peter, 1986; King and Millar, 1997]. In general, these findings were, however, over simplified and incomplete, in that not all the endogenous forms of GnRH have been tested using comparable techniques [Peter *et al.*, 1985; Licht *et al.*, 1987; Millar *et al.*, 1989; Habibi *et al.*, 1992]. This will be discussed in light of the cloning of the non-mammalian receptors, in more detail in chapters 2, 4 and 5.

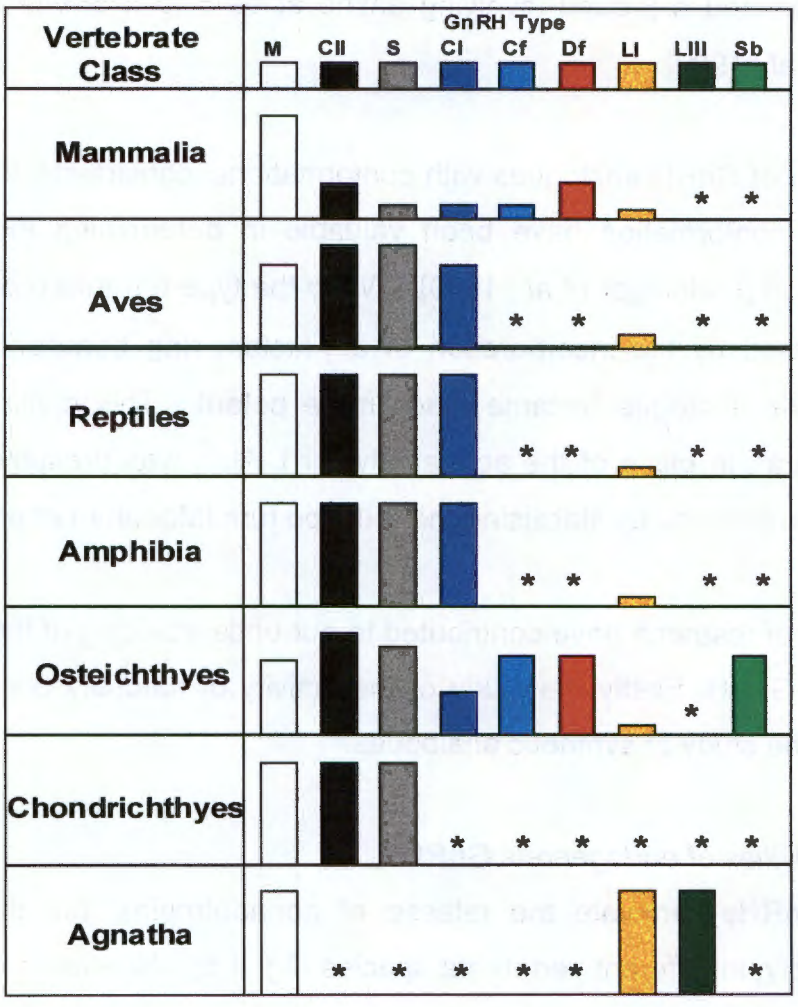


Figure 1.5. Activities of some of the naturally occurring GnRHs in vertebrates, derived from King and Millar [1997]. Activity is shown from 0-100%, as summarised from studies on gonadotropin release from pituitary cells and receptor binding assays. * Represents not determined.

1.8.3 The individual amino acids and GnRH activity

The entire peptide is required for gonadotropin-releasing activity, as any truncations of GnRH result in a loss of activity [Sandow *et al.*, 1978]. The conservation of the NH_2 and COOH terminal domains throughout evolution indicates their importance in GnRH activity and function. While the variation in residues 5 to 8 may suggest that they are not important in GnRH activity, it may imply that they are important for receptor/ligand selectivity. The development and understanding of GnRH analogues, including both agonists and antagonists has been vital for understanding the importance of individual amino acids in GnRH activity [for reviews, see Rivier *et al.*, 1981; 1992].

The NH_2 terminal domain

$pGlu^1$ is required for biological activity. Most substitutions at this position caused a loss of activity except analogues with substitutions resembling the pGlu structure, such as the cyclic $(O=)Ser^1$ [Fujino *et al.*, 1972], D-pGlu [Hirotsu *et al.*, 1974], acylated Gly^1 and formyl Gly^1 [Okado *et al.*, 1973]. Despite the similarity between $pGlu^1$ and Pro, however, substitutions by this amino acid, resulted in a loss of activity [Fujino *et al.*, 1972]. $pGlu^1$ may also be important for receptor activation as demonstrated by its substitution in antagonists [for reviews, see Rivier *et al.*, 1981; 1992].

Several substitutions in position 2 of GnRH have indicated the importance of an aromatic amino acid in this position. This is confirmed by the high activity of $[Trp^2]$GnRH [Monohan *et al.*, 1973] and the relatively low activity of other substitutions [for reviews, see Karten and Rivier, 1986; Sealfon *et al.*, 1997]. Further support for the importance of an aromatic amino acid at this position comes from the recent isolation of $[Tyr^2,Val^7]$GnRH (guinea pig GnRH) [Jimenez-Liñan *et al.*, 1997]. This change in GnRH may, however, be accommodated by changes in the guinea pig GnRH receptor. Bulky hydrophobic amino acid residues have replaced His^2, resulting in antagonistic activity. Recently His^2 has been suggested to interact directly with Lys^{121} [Zhou *et al.*, 1995] and possibly Asp^{98} [Rodic *et al.*, 1996] of the human GnRH receptor. His^2 may, therefore, be important for direct interaction with the receptor, as well as being important for the intrinsic activity of the molecule [Vale *et al.*, 1972].

Substitution studies have indicated the critical role, of an aromatic amino acid in position three of GnRH in receptor activation. This is also confirmed by the natural substitution of Trp^3 by Tyr^3 in lamprey GnRH (fig 1.1) [Powell *et al.*, 1996]. Studies with the unnatural aromatic amino acids, 2-naphthyl-Ala and pentamethyl-Phe (Me5-Phe), resulted in analogues with approximately half the activity [Sandow *et al.*, 1978; Coy *et al.*, 1974]. Since the only common feature of these amino acids is their ability to form π-π interactions with certain aromatic compounds, Trp^3 possibly interacts with an aromatic amino acid residue of the receptor.

Despite the total conservation of Ser^4 in GnRH, substituted analogues at this position are relatively active [Sandow *et al.*, 1978; for review, see Coy *et al.*, 1975(a)]. The size of the substituted side chain, however, appears to be important, with larger substitutions such as Leu or Ser(But) decreasing activity [Sandow *et al.*, 1978]. The high conservation and spatial constraints at this position may imply that Ser^4 occupies the binding pocket, where it may play a role as a Hydrogen-bond donor with the receptor [for review, see Sealfon *et al.*, 1997].

The central variable domain

As implied by the high variability with endogenous GnRH peptides including Tyr, His, Leu, and Asp, substitutions at position 5 show a high tolerance, with only small loses in GnRH activity [Sandow *et al.*, 1978; for review, see Coy *et al.*, 1975(a)].

Gly^6 is conserved in all the vertebrate GnRHs except the ancient Lamprey and the Tunicate isoforms. As discussed above, the presence of an achiral amino acid at this position is vital in supporting the biologically active conformation of GnRH, which requires a β II-type turn [Momany, 1976(a)]. D-amino acids enhance this conformation and thus have a higher activity [Monohan *et al.*, 1973]. The Cys in position 6 of tunicate GnRH II [Leu^5,Cys^6,His^7,Ala^8]GnRH (fig 1.1) may result in the formation homodimers, via the formation of a disulphide bridge [Powell *et al.*, 1996]. This may be important for decreasing enzymatic degradation.

Most substitutions of Leu^7, did not decrease the activity of GnRH, as suggested by the large variability of the natural GnRHs at this position. Analogues with small sidechains, such as Ala and Gly, or charged sidechains, such as Lys and Arg, were however, less active [Sandow *et al.*, 1978]. Thus large uncharged amino acids may be required at this position, as confirmed by the demonstration of the increase in LH-releasing activity of [Trp^7]GnRH in sheep [Millar *et al.*, 1989].

Arg^8 is important for high affinity binding of mGnRH to its receptor [Millar and King, 1983; Millar *et al.*, 1986(b); Millar *et al.*, 1989]. The activity for several analogues, with substitutions at this position have been studied, and conclude that a basic amino acid at this position is required for activity [Hazum *et al.*, 1977; Millar and King, 1983; for review, see Coy *et al.*, 1975(a)]. In recent studies the acidic Glu^{301} in extracellular

loop III of the mouse GnRH receptor was shown to confer specificity for Arg^8 GnRH [Flanagan *et al.*, 1994]. The non-mammalian receptors do not appear to be selective for Arg^8 GnRH. It was, therefore, proposed that they may not have an acidic amino acid in the corresponding position of extracellular loop III. This will, however, be discussed in greater detail in later chapters.

The conserved COOH-terminal domain

The rigidity imposed by the Pro in position 9 and its high conservation, suggest that it may be very important for both the structure and activity of GnRH. The substitutions, which have been tested decreased activity [Sandow *et al.*, 1978].

The Gly-NH_2 terminus, however, can be altered considerably without much change in activity, with substitutions such as Ala^{10} [Fujino *et al.*, 1972] retaining 10% of activity. Potent analogues of GnRH, with prolonged activity have been made by replacing the COOH-terminal glycine amide with an ethylamide [Arimura *et al.*, 1974] and 2,2,2-trifluroethylamide [Coy *et al.*, 1975(b)].

1.9 The GnRH receptor

The initial cloning of the mouse GnRH receptor by PCR [Tsutsumi *et al.*, 1992] and subsequent cloning by expression in *Xenopus* oocytes [Reinhart *et al.*, 1992] and a mammalian cell line [Perrin et al., 1993] and cloning of the other mammalian receptors by several groups, including human [Kakar *et al.*, 1992; Chi *et al.*, 1993], rat [Eidne *et al.*, 1992; Kaiser *et al.*, 1992], ovine [Illing *et al.*, 1993; Brookes *et al.*, 1993], bovine [Kakar *et al.*, 1993], and porcine [Weesner and Matteri, 1994], led to the classification of the mammalian GnRH receptors as members of the G-protein coupled receptor (GPCR) superfamily.

1.9.1 General characteristics of G-protein coupled receptors

GPCRs represent the largest family of proteins, with over 300 known members, which have been cloned and characterised [for reviews, see Dohlman *et al.*, 1991; Baldwin, 1993; Strader *et al.*, 1994; 1995 and Van Biesen *et al.*, 1996]. These receptors are grouped by their characteristic coupling to heterotrimeric GTP-binding proteins (G-proteins). They are important in conveying extracellular signals to the intracellular milieu of target cells, causing an appropriate response. GPCRs have

been classed into three major groups according to their sequence alignment: the large rhodopsin-like family, the secretin receptor group and the metabotropic glutamate receptors. They respond to a diverse array of signaling molecules ranging from photons of light hitting the retina, odorants, neurotransmitters, cations, lipids, cytokines, glycoprotein hormones, to peptides. Despite their diverse signaling mechanisms they have considerable homology.

The most characteristic feature of G-protein coupled receptors are the 7 hydrophobic α-helical domains, which traverse the membrane, and are linked by alternating extracellular and intracellular loops. The transmembrane helices have the highest amino acid homology between receptors of the same and different subgroups. The two-dimensional electron crystallography of the bovine rhodopsin receptor, confirmed the hydrophobicity prediction of the arrangement of the 7 α-helical transmembrane domains [Schertler *et al.*, 1993]. The transmembrane helices range in size from 18 amino acids, which is the minimum number of residues required to span the cell membrane in an α-helical conformation, to 25 amino acids. Based on the assumption that the hydrophobic nature of the membrane will exclude any hydrophilic residues, the transmembrane domains can be assigned [Kyte and Doolittle, 1982; Chou and Fasman, 1978]. The positively charged Arg and Lys residues, when they occur on the cytoplasmic side of a transmembrane helix, may be predicted to lie outside the membrane by the hydrophobicity plot. These residues should, however, be incorporated into the transmembrane domain [Ballesteros and Weinstein, 1992]. They may in fact mark the boundary of the cytoplasmic end, by their ionic interaction with the negatively charged headgroups of the inner cell membrane [for review, see Ballesteros and Weinstein, 1995].

The extracellular and intracellular loops have a large variation both in size, ranging anywhere between 5 and 420 amino acids [Baldwin, 1993], and sequence. The extracellular loops along with the transmembrane helices are thought to be important for ligand recognition [for reviews, see Dohlman *et al.*, 1991; Strader *et al.*, 1994; Savarese and Fraser, 1994]. The intracellular loops, especially intracellular loop III are thought to be important for interaction with G-proteins, and may convey the specificity of this interaction. There are a large number of G-proteins, and GPCRs

seem to be specific in interacting with particular subtypes [for reviews, see Spiegel *et al.*, 1992].

Characteristic postranslational modifications to G-protein coupled receptors include N-linked glycosylation of the N-terminal domain [Rands *et al.*, 1990; Davidson *et al.*, 1995], disulphide bridge formation between extracellular loops II- and III [Dohlman *et al.*, 1990] and the palmitoylation of cysteine residues in the carboxy-terminal domain [O' Dowd *et al.*, 1989]. These modifications, may be important for optimal receptor expression, receptor conformation and G-protein interaction [for review, see Strader *et al.*, 1994]. The phosphorylation of intracellular serine/threonine residues, may be important in receptor desensitisation [Lefkowitz *et al.*, 1990].

1.9.2 Structure and function of the mammalian GnRH receptors

The mammalian GnRH receptor is a member of the rhodopsin family of GPCRs. It has, however, several features, which are uncharacteristic of this class of GPCRs, which will be discussed in detail in the following sections. Analogous to the studies on endogenous GnRH peptides, alignment of GPCRs has been invaluable in the study of the structure and function of these receptors, and has helped identify several sites important for structural integrity, ligand binding, agonist induced internalisation and G-protein coupling [Probst *et al.*, 1992]. Further dissection of the relevance of these conserved/unconserved sites has been possible with the aid of site-directed mutagenesis. Although the results of such studies are complicated by the affect of the mutation on receptor expression, many valuable conclusions have been made.

Postranslational processing of the mammalian GnRH receptors

The rodent GnRH receptors have three glycosylation consensus sites, two in the amino-terminus and one in the extracellular loop I. The other mammalian GnRH-Rs have, however, only one potential site in the amino-terminus, and the other site in extracellular loop I. These sites were investigated in the mouse GnRH receptor, using a combination of site directed mutagenesis and photo-affinity labelling [Davidson *et al.*, 1995; Arora *et al.*, 1997]. Only the two amino-terminal sites were implicated. These two sites, when mutated, resulted in receptors with a lower expression, due to either decreased stability, membrane trafficking or expression. The binding affinity and signal transduction were, however, unaltered. The human

GnRH receptor has only one glycosylation site at Asn^{18} in the amino-terminus. When an additional site was introduced into the amino-terminus by site directed mutagenesis, the receptor expression was increased [Davidson *et al.*, 1996(a)].

Disulphide bridges between Cys residues create an internal scaffold within the receptor, stabilising the receptor conformation. There is a conserved disulphide bridge between Cys residues in extracellular loops I and II, which is present in most GPCRs [for review, see Strader *et al.*, 1994]. Disulphide bridges may be important for high affinity binding of GnRH because treatment with reducing agents decreases the affinity [Keinan and Hazum, 1985]. This disulphide bridge was demonstrated between Cys^{104} and Cys^{196} of the human GnRH receptor [Davidson *et al.*, 1997] and Cys^{114} and Cys^{195} of the rat GnRH receptor [Cook and Eidne, 1997]. An additional disulphide bridge between the amino-terminus Cys^{14} and Cys^{200} in extracellular loop II, has also been demonstrated in the human GnRH receptor [Davidson *et al.*, 1997], but there is conflicting evidence for the occurrence of this disulphide bridge in the rat GnRH receptor [Cook and Eidne, 1997]. Cys^{14} appears to be involved in the covalent binding to the photoreactive side chain of [azidobenzoyl-d-Lys^{6}]GnRH resulting in the irreversible activation of the receptor [Davidson *et al.*, 1997]. The interaction between the side chain and Cys^{14} may, therefore, keep the photoreactive ligand in the correct orientation for receptor activation. However, mutants of Cys^{14} still exhibit reduced ligand binding and receptor activity [Cook and Eidne, 1997]. This interaction as well as the disulphide bridge between Cys^{14} of the NH_2-terminus and Cys^{200} of extracellular loop II may, therefore, not be vital for endogenous GnRH binding, but the disulphide bridge between extracellular loops I and II is essential for receptor function [Davidson *et al.*,1997; Cook and Eidne, 1997] (fig. 1.6).

Transmembrane helices

In the mammalian GnRH receptors transmembrane helices (TM) II and VII are the most conserved (96%), followed by TM III (93%), TM VI (80%), TM V (78%) TM I (70%) and TM IV (58%). A number of highly conserved amino acid residues in GPCRs occur in the transmembrane helices, including Pro residues in TM II and TM IV – TM VII, which are thought to produce kinks in the α-helices [Baldwin, 1993]. Other conserved residues in TM helices include Asn^{53} (TM I), Trp^{164} and Ser^{167} (TM

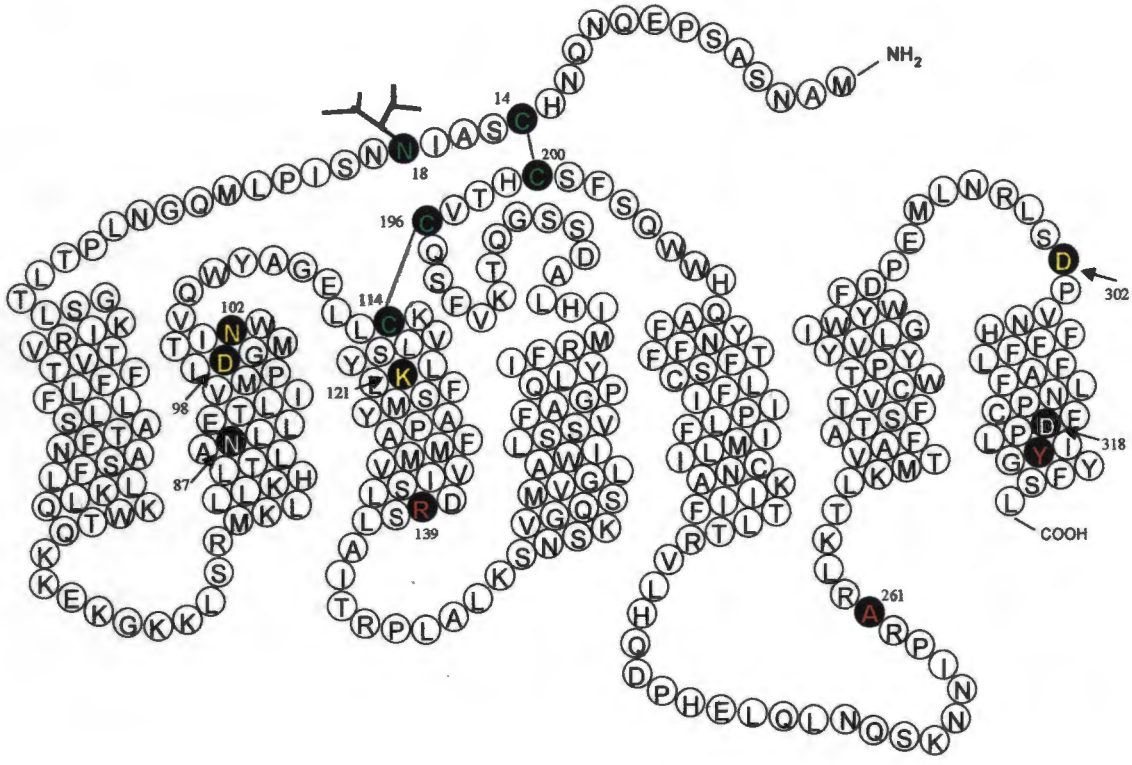


Figure 1.6. Diagram showing the amino acid sequence of the human GnRH receptor and the putative two-dimensional arrangement of the seven transmembrane helices. Residues referred in the text are numbered. Residues involved in postranslational modifications (green), ligand binding (blue) G-Protein interaction (red) and interhelical interactions (white) are shown.

V), and Asn^{315} and Tyr^{323} (TM VII) [for review, see Davidson *et al.*, 1994(a)]. Asn^{87} and Asp^{318} in TMs II and VII respectively, appear to have undergone a reciprocal exchange when compared to other GPCRs. Mutagenesis experiments of these two residues suggest that the reciprocal change seen in the mammalian GnRH receptors can be accommodated by the close association of TM helices II and VII [Zhou *et al.*, 1994]. These mutagenesis experiments also suggested a possible role for Asp^{318} in receptor signal transduction [Zhou *et al.*, 1994].

The Ligand Binding Site

Mutagenesis studies have been vital in understanding the ligand binding site of the receptor. Several GPCRs have been studied and the binding sites appear to be located in the TM domains of the receptors [for reviews, see Strader *et al.*, 1994; Schwartz, 1994; Strader *et al.*, 1995].

Asp^{113} of the β-adrenergic receptor in TM III has been shown to form a salt bridge with the amine group of the ligand. Mutagenesis studies have confirmed its role in ligand binding. Lys^{121} occurs at an analogous position in the human GnRH receptor. Mutation of Lys^{121} resulted in a loss of agonist affinity, while high affinity antagonist binding was retained [Zhou *et al.*, 1995]. GnRH agonists and antagonists differ substantially in the amino-terminal region, thus implicating this region for interaction with Lys^{121}. This interaction may involve the imino group of His^2 [Zhou *et al.*, 1995]. Asp^{98}, is also thought to interact with His^2 of GnRH, as mutagenesis of this site had little effect on GnRH analogues with substitutions at position two [Rodic *et al.*, 1996].

Asn^{102}, which is located at the top of TM II, has been shown to interact with the glycine amide of GnRH [Davidson *et al.*, 1996(b)]. Combined mutagenesis and GnRH analogue studies involving substitutions at the COOH-terminal glycine amide were utilised.

Mutation of Glu^{301} in extracellular loop III of the mouse GnRH receptor to a neutral Gln, resulted in the loss of selectivity for GnRH analogues with the positively charged Arg at position 8 [Flanagan *et al.*, 1994]. The Gln^{301} mutant receptor, however, showed no loss in affinity for conformationally constrained analogues [Flanagan *et al.*, 1994]. This implies that Glu^{301} in the mouse- or Asp^{302} in the human receptor may interact electrostatically with Arg^8 of GnRH, inducing GnRH to assume the active conformation [Flanagan *et al.*, 1994].

Thus, GnRH binding to the human GnRH receptor appears to involve Lys^{121} in TM III, Asn^{102} and Asp^{98} in TM II and Asp^{302} in TM VII. While Lys^{121} docks the amino-terminus, possibly His^2, Asp^{98} interacts with His^2, Asn^{102} may bind to the glycine amide, and Asp^{302} may interact with Arg^8, inducing GnRH to adopt the active conformation (fig. 1.6).

G-Protein interaction and signal transduction

Mutagenesis and chimeric receptor studies for different GPCRs have implicated the intracellular domains, particularly the second and third intracellular loops (IL) in G-protein interactions [for reviews, see Dohlman *et al.*, 1991; Savarese and Fraser, 1992; Strader *et al.*, 1994].

Alignment of GPCRs led to the identification of the highly conserved DRY motif, which is located at the border of TM III and IL II. The Asp and Arg residues have previously been shown to be important in G-protein coupling [for review, see Savarese and Fraser, 1992]. Uncharacteristically the conserved Tyr of this motif is a Ser in the cloned mammalian GnRH receptors. Mutational analysis of this motif implicated the Asp^{138} and Arg^{139} residues as important contributors of receptor expression, agonist induced activation and internalisation. Ser^{140}, however, is unimportant [Davidson *et al.*, 1994(a); Arora *et al.*, 1997]. The Arg residue has been shown to interact with Asp^{138} and Ile^{143}, which would occur one turn away if intracellular loop II forms a α-helix [Ballesteros *et al.*, 1998]. These residues are thought to 'cage' the Arg appropriately for the active and inactive receptor conformations [Ballesteros *et al.*, 1998].

Another uncharacteristic feature of the mammalian GnRH receptors is the GPCR conserved $NPX_{2-3}Y$ motif in TM VII. In the GnRH receptors this motif is DPLIY (Asp^{318}, Pro^{319}, Leu^{320}, Ile^{321}, Tyr^{322}). The Asn is changed to an Asp, but the Pro and Tyr residues are conserved. Mutagenesis studies revealed the importance of Asp^{318} in signal transduction [Zhou *et al.*, 1993]. Mutations of Tyr^{322} showed the requirement of an aromatic amino acid at this position in the receptor, in receptor activation and signal transduction [Arora *et al.*, 1996].

Several residues in IL III of GPCRs have been mutated, rendering the receptor constitutively active. An example of such a mutation is, Ala^{293} of the α1B-adrenergic receptor, which when mutated to any other amino acids, results in a receptor, which is contitutively active [Allen *et al.*, 1991]. When Ala^{261} in the analogous position in the human GnRH receptor is mutated to an amino acid with a bulky side chain, the resulting receptor is unable to activate the G-protein, and is thus uncoupled [Myburgh *et al.*, 1998].

Receptor desensitisation and internalisation

One of the most remarkable features of the mammalian GnRH receptors is the absence of an intracellular COOH-terminal tail. The COOH-terminal tail of GPCRs has been shown to play an important role in homologous desensitisation, possibly via receptor phosphorylation [for review, see Lefkowitz *et al.*, 1990]. The GnRH receptor does not show rapid homologous desensitisation in α-T3 cells [Davidson *et al.*, 1994(b); McArdle *et al.*, 1995]. The GnRH receptor does, however, show a long term desensitisation to continuous GnRH stimulation [for review, see Clayton, 1989]. This long-term desensitisation is both Ca^{2+} [Smith and Conn, 1983] and inositol phosphate independent [Hawes and Conn, 1992] and may result in GnRH receptor expression being down regulated either by decreased efficiency of receptor mRNA translation [Tsutsumi *et al.*, 1995] or regulation of gene expression [Lerrant *et al.*, 1995]. The lack of short-term homologous desensitisation may be a critical feature of the GnRH receptor, which enables it to respond to a pulsatile stimulation of GnRH, ultimately leading to the LH-surge. Studies on a chimeric GnRH receptor with the cytoplasmic tail of thyrotropin-releasing hormone (TRH) receptor revealed that the lack of a cytoplasmic COOH-terminal tail in the GnRH receptor can account for part of the receptor's inability to undergo homologous desensitisation to short-term stimulation with GnRH [Heding *et al*, 1998].

The presence of a COOH-terminal cytoplasmic tail may also be required for agonist-stimulated receptor internalisation. This has been demonstrated for the TRH receptor [Nussenzveig *et al.*, 1993], and will be discussed in greater detail in later chapters of this thesis. G-protein coupling and agonist-induced receptor internalisation appear to be closely linked, where mutations that affect coupling often, affect internalisation. Mutations of Ala^{261} to amino acids with bulky side chains, which were uncoupled, showed a reduced ability to internalise [Myburgh *et al*, 1998]. Mutations of Asp^{138} and Arg^{139} of the DRY/S motif at the border of TM III and IL II also exhibit reduced internalisation [Arora *et al.*, 1997].

1.9.3 Expression and regulation of the GnRH receptor

The mammalian GnRH receptors have a widespread distribution in both reproductive and non-reproductive tissues. GnRH receptors have been characterised in the pituitaries and brain [Millan *et al.*, 1986] of several species [for review, see Stojilkovic

et al., 1994]. GnRH receptors have also been localised in the Leydig cells of the testis [Bourne *et al.*, 1980; Millar *et al.*, 1982], and the granulosa and luteal cells of the ovary [Harwood *et al.*, 1980; Hazum and Nimrod, 1982; Seguin *et al.*, 1982]. GnRH binding sites have been found in human breast, [Eidne *et al.*, 1985; 1987], endometrial and ovary carcinomas [Imai *et al.*, 1994(a); 1994(b)]. GnRH receptor mRNA has also been identified in non-reproductive tissues, by reverse transcriptase PCR [Kakar and Jennes, 1995].

The human GnRH receptor has been localised to chromosome 4q13.2-13.3 [Morrison *et al.*,1994]. The receptor is encoded by a 18.9 kb gene, which like the mouse GnRH receptor gene [Zhou and Sealfon, 1994], consists of three exons [Fan *et al.*, 1994]. The GnRH receptor appears to be regulated by several different factors and expression may vary greatly during the estrous cycle [Clayton *et al.*, 1980; Marian *et al.*, 1981; Kaiser *et al.*, 1993]. The GnRH receptor gene is under the homologous regulation of GnRH, as pulses of the hormone increase receptor expression, while a continuous administration of GnRH shows no change in receptor mRNA levels [Kaiser *et al.*, 1993; Bauer-Dantoin *et al.*, 1995; Sakakibara *et al.*, 1996]. This increase in receptor expression may be as a result of estrogen [Bauer-Dantoin *et al.*, 1995]. Gonadal steroids such as estrogen as well as inhibin [Sealfon *et al.*, 1990] appear to increase the GnRH response [Clayton *et al.*, 1980; Duncan *et al.*, 1986; Quinones-Jenab *et al.*, 1996]].

The mouse [Albarracin *et al.*, 1994; Duval *et al.*, 1997(a)], and human [Fan *et al.*, 1995; Kakar, 1997] GnRH receptor gene promoters have been characterised [for reviews, see Eidne, 1994; Millar, 1997]. Both the mouse and human GnRH receptor promoters have multiple regulatory elements such as, Pit-1 (pituitary specific), AP-1 and AP-2 (protein kinase responsiveness), SF-1 (steroidogenic factor), TRE (thyroid hormone response element), PRE/GRE (progesterone/glucocorticoid response element), and CRE (cAMP response element) [for review, see Millar, 1997]. The mouse GnRH receptor promoter has a novel GRAS (GnRH receptor activity sequence), which is the recognition sequences for a tripartite, cell specific enhancer [Duval *et al.*, 1997(b)]. Regulation of the GnRH receptor may, therefore be a central mechanism in the complex signaling system regulating reproductive pathways.

1.10 Concluding remarks

GnRH has been highly conserved throughout vertebrate evolution (some 500 million years), and plays a central role in the regulation of reproduction. Since its discovery in 1971 by Drs Schally and Guillemin, GnRH has been widely used as a therapeutic agent for reproductive disorders. Due to the nature of the action of GnRH, it is utilised to both restore fertility and to selectively suppress the pituitary gonadal axis. In order to restore fertility GnRH analogues are administered in a pulsatile manner so as to mimic the biological release of GnRH. This is used for the treatment of Kallmann's syndrome, pituitary tumours and hypogonadotropic hypogonadism. If GnRH is administered continually, by long acting GnRH analogues, the pituitary gonadal axis becomes suppressed via the down regulation of the GnRH receptor. This can be used to treat endometriosis, uterine fibroids and prostate cancer, where normal pituitary function must be suppressed [for review, see Conn and Crowley, 1990]. The development of long-acting, enzyme resistant analogues would be useful for such therapies. Most of the therapeutic agents are confined to injectable preparations, thus the design of orally active (non-peptide) analogues would have a great impact in furthering the applications of GnRH-related therapies. An additional use of GnRH in this regard, includes antifertility agents or contraceptives. These would be more specific than the steroidal contraceptives, thus, having fewer side effects.

The cloning of the mammalian GnRH receptors has been important for the delineation of ligand/receptor interactions. Several residues in the receptor have been identified as important for ligand recognition and receptor activation. A further understanding of these interactions may help in the design of therapeutic agents, as described above. The mammalian GnRH receptors are highly conserved (>80% amino acid identity), thus the analysis of sequences from more evolutionarily distinct vertebrates which recognise the same ligand may be useful in determining both agonist and antagonist binding sites in the receptor. The recent cloning of the catfish GnRH receptor by Tensen and co-workers [1997] has already proved useful in this regard. This thesis describes the cloning and characterisation of GnRH receptors from the amphibian, *X. laevis* and the teleost, *Carassius auratus.* The primary structure and characterisation of ligand selectivity provides some useful foundations for future work towards understanding ligand recognition in the GnRH receptor. The

description of multiple receptor subtypes in the goldfish and possibly in *X. laevis* also provides valuable information into alternative roles of GnRH and its receptor. The co-occurrence of multiple GnRH receptors and ligands implicates a more diverse role of GnRH, which we are only beginning to understand.

Molecular Cloning, Expression and Characterisation of a Gonadotropin-Releasing Hormone Receptor from *Xenopus laevis* Pituitary

2.1 Summary

Mammalian GnRH ([Arg^8]GnRH, mGnRH) and chicken GnRH II ([His^5, Trp^7, Tyr^8]GnRH, cGnRH II) have been shown to be present in amphibia. mGnRH, which predominates in the preoptic area, is thought to act as the major hypophysiotropic factor, while cGnRH II, which has a widespread distribution, but predominates in the hindbrain, may have a role in neuromodulation or neurotransmission. A full-length cDNA for a putative gonadotropin-releasing hormone (GnRH) receptor was cloned from *Xenopus laevis* pituitary cDNA, using PCR-based methodology. The 1.1 kbp clone is encoded by an approximately 11.4 kbp gene containing two introns. The cDNA encodes a receptor of 368 amino acids with the features of a typical G-protein coupled receptor. The amphibian receptor has a 46% amino acid identity to the human GnRH receptor [Kakar *et al.*, 1992; Chi *et al.*, 1993] and a 55% amino acid identity to the catfish GnRH receptor [Tensen *et al.*, 1997]. Unlike the mammalian GnRH receptors, but like the catfish GnRH receptor, the amphibian receptor has an intracellular carboxy-terminal tail [for review, see Sealfon *et al.*, 1997]. The receptor was transiently transfected into COS-1 cells and inositol phosphate response monitored to characterise ligand specificity. Although previously mGnRH was reported to be equipotent to other naturally occurring GnRH subtypes in stimulating LH release from the amphibian pituitary, cGnRH II was found to be 2000-fold more potent than mGnRH. This contrasts with the mammalian GnRH receptors, which show a 10-fold preferential binding of mGnRH compared to cGnRH II. The differences in ligand selectivity are presumably a result of differences in amino acids making up the binding sites or alternatively, indirectly altering the conformation of the receptor such that the binding pocket is altered. This receptor thus offers the potential for delineating important conformational residues at a molecular level.

2.2 Introduction

From an evolutionary perspective amphibians are progenitors of mammals via a mammal-like reptile and may, therefore, be considered to represent the primitive mammalian condition [Carter, 1967]. Amphibians are a useful model system for the study of the mammalian endocrine system, as the structures of the major regulatory components such as GnRH, thyrotropin-releasing hormone (TRH), corticotropin-releasing factor (CRF), growth hormone-releasing hormone (GHRH) and somatostatin are conserved. Amphibians like mammals have the mammalian and chicken II isoforms of GnRH [King and Millar, 1986; Dellovade *et al.*, 1993; King *et al.*, 1994(b); Kasten *et al.*, 1996; Lescheid *et al.*, 1997; White *et al.*, 1998 and for reviews, see King and Millar 1995; 1997; Sherwood *et al.*, 1997]. The distribution of these peptides in amphibia has been intensely studied. While the general pattern of distribution between the amphibian genera, may differ slightly, the overall pattern seems to be conserved. mGnRH, which predominates in the preoptic area, with fibres extending into the hypothalamus and median eminence, appears to be the central hypophysiotropic regulator [di Meglio *et al.*, 1991; Fasano *et al.*, 1993; Muske *et al.*, 1994; Licht *et al.*, 1994; King *et al.*, 1994(a); Collin *et al.*, 1995; Di Matteo *et al.*, 1996; Pinelli *et al.*, 1997]. cGnRH II, however, appears to have a more widespread distribution and has a two-fold higher concentration than mGnRH in the brain [Licht *et al.*, 1994]. cGnRH II distribution does overlap with mGnRH distribution in the preoptic and hypothalamic areas, but mGnRH predominates in these areas, by approximately 5-fold [Licht *et al.*, 1994]. cGnRH II predominates in the hindbrain, particularly the medulla oblongata, telecephalon and spinal cord, which supports its putative role as a neuromodulator [King *et al.*, 1994(a); Licht *et al.*, 1994; Muske *et al.*, 1994; Collin *et al.*, 1995; Di Matteo *et al.*, 1996].

Although several groups have reported the presence of salmon GnRH (sGnRH) in amphibia using immunocytochemistry [Fasano *et al.*, 1993; Di Matteo *et al.*, 1996] this remains to be confirmed. Others have detected an unknown peptide with similar properties to sGnRH, but which does not show complete identity with sGnRH-specific antisera [Sherwood *et al.*, 1986; Cariello *et al.*, 1989; King *et al.*, 1994(a)]. If sGnRH or a third unidentified form of GnRH with properties similar to sGnRH, is present, it does not, however, appear to be involved as a hypophysiotropic factor [Fasano *et al.*,

1993]. A third form of GnRH, namely [Trp^8]GnRH, has recently been cloned from *Rana dubowski* [H. Kwon, University of Korea, unpublished]. In this species, mGnRH has not yet been identified and thus, this alternate form, may replace mGnRH, or may represent a third form of GnRH present in this species. Interestingly, this peptide ([Leu^7, Trp^8]GnRH, may have similar chromatographic properties to sGnRH ([lTrp^7, Leu^8]GnRH) and may be the third form of GnRH present in amphibia, which co-elutes with sGnRH in many species [Sherwood *et al.*, 1986; Cariello *et al.*, 1989; King *et al.*, 1994(a)].

The cloning of the mammalian GnRH receptors has been valuable for the delineation of amino acid residues important in ligand binding, G-protein interaction and interhelical interactions. The exploitation of structural/functional evolution of GnRH receptors in other vertebrates, may help in this endeavour. The sequence of an evolutionarily distinct GnRH receptor, which responds to the same ligand may be helpful in furthering our understanding of structure-activity domains of GnRH receptors. We have, therefore, determined the sequence of the *Xenopus laevis* pituitary GnRH receptor and characterised its selectivity for different GnRH agonists.

Amino acid residues which are conserved between the amphibian receptor and the mammalian receptors may be important for the receptor conformation, G-protein coupling or ligand induced receptor activation. Despite the similarities between amphibians and mammals, the amphibian pituitary GnRH receptor is, however, likely to be substantially different from several aspects. Firstly the mammalian receptor shows a high fidelity for mGnRH and a substantially lower affinity for cGnRH II and other GnRH isoforms, while the amphibian pituitary has been reported to not discriminate between different GnRH isoforms [Licht *et al.*, 1984; 1987]. Secondly the mammalian pituitary requires a pulsatile input of GnRH to facilitate LH and FSH secretion, while the amphibian pituitary responds well to the chronic administration of GnRH [Licht and Potter, 1986]. GnRH receptor regulation and desensitisation may, therefore, be substantially different between the two vertebrates.

The cloning and characterisation of the *X. laevis* pituitary GnRH receptor, described in this chapter, however, reveals that these early reports are not correct. Firstly the amphibian receptor is highly selective for cGnRH II compared to mGnRH. The amphibian receptor also shows partial stimulation with a mammalian GnRH receptor antagonist. These differences in the pharmacology between the mammalian- and the amphibian GnRH receptors may be dictated by the differences in the primary structure of the receptors. The amphibian receptor also has a carboxy-terminal tail, which is absent in the mammalian GnRH receptors. This may imply an alternative mechanism of regulation and desensitisation.

2.3 Materials and Methods

2.3.1 GnRH analogues

The following GnRH analogues were used: Mammalian GnRH; [His^5, Trp^7, Tyr^8]GnRH (cGnRH II); [Trp^7, Leu^8]GnRH (sGnRH); [Gln^8]GnRH (cGnRH I); [T-BuSer^6, Pro^{9}NHEt]GnRH (buserelin); [His^5]GnRH; [Trp^7, Tyr^8]GnRH; [His^5, D-Tyr^6]GnRH; [His^5, D-Arg^6, Trp^7, Tyr^8]GnRH; [His^5, D-Trp^6, Trp^7, Tyr^8]GnRH; [His^5, D-Lys^6, Trp^7, Tyr^8]GnRH; [D-Ala^6, N-Me-Leu^7, Pro^9-NHEt]GnRH; [Ac-*D*-Nal^1, *D*-4-ClPhe^2, *D*-Pal^3, Ile^5, *D*-IsopropylLys^{6,8}, *D*-Ala^{10} NH_2]GnRH (antagonist 135-18); and [Ac-*D*-p-ClPhe^{1,2}, *D*-Trp^3, *D*-Lys^6, *D*-Ala^{10} NH_2]GnRH (antagonist 26) were prepared by solid-phase synthesis and purified by C-18 reversed-phase chromatography.

2.3.2 Tissue dissection and isolation of total RNA and genomic DNA

Adult male African clawed frogs were decapitated, and the cranial bone cut away to expose the brain and pituitary. The pituitaries from 10 frogs were pooled for RNA extraction. Total RNA was prepared by extraction with guanidinium thiocyanate [Chomczynski and Sacchi, 1987]. The dissected tissues were placed in 500 µl of denaturing solution (4 M guanidinium thiocyanate; 25 mM sodium citrate, pH7; 0.5% sarcosyl; 0.1% 2-mercaptoethanol), frozen on dry ice and stored at -70^{0}C until required. Tissues were homogenised prior to extraction using a sterile 2 ml syringe with a 0.8 x 40 mm needle (Promex). RNA was visualised on a 1% formaldehyde-agarose gel. To minimise RNA degradation by ribonucleases during extractions, solutions were treated with 0.1% diethylpyrocarbonate (DEPC), prior to autoclaving.

solutions which could not be treated with DEPC, were made up with DEPC treated distilled water. All glassware, newly opened microfuge tubes and pipette tips were autoclaved.

DNA was extracted from five livers. Once dissected the livers were placed on ice in a sterile 50 ml Falcon tube in 10 x volume of lysis buffer (0.6% SDS; 20 mM Tris-HCL, pH 7.5; 100 mM NaCl; 50 mM EDTA, pH 8.0). The tissue was homogenised using a Polytron homogeniser and incubated at 37^{0}C for 30 minutes. Proteinase K was added to a final concentration of 0.4 mg/ml, and the tissue incubated for a further five hours at 37^{0}C. The DNA was extracted with an equal volume of equilibrated phenol (pH 8.0), phenol:chloroform:*iso*-amylalcohol (50:49:1, *v/v*) and chloroform:*iso*-amylalcohol (49:1, *v/v*), by mixing for 15 minutes, followed by centrifugation at 4000 g for 10 minutes to separate the phases. DNA was precipitated with two volumes of 100% ethanol and 0.3 M sodium acetate (pH 5.2) and collected by spooling with a glass rod. Once collected the DNA was dipped into 70% ethanol and air dried. DNA was resuspended overnight at 4^{0}C in Tris-EDTA buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA, pH 8.0). The absorbance at 260 nm was determined and the DNA diluted to a final concentration of 1 mg/ml. DNA was checked for degradation on a 1% TBE (0.09 M Tris-borate; 0.002 M EDTA, pH 8.0) agarose gel containing ethidium bromide (50ng/ml) and visualised with UV light.

2.3.3 Degenerate Primer PCR

Degenerate primers were designed to regions conserved in the cloned mammalian GnRH receptors [personal communications with Dr Janet Hapgood]. The location of the primers with regard to the mouse GnRH receptor gene can be seen in figure 2.1. The degenerate primers were as follows (N = g, a, t, or c; r = g or a; y = t or c; x = c or a, h = a, c, or t; d = a, g, or t):

JH1s 5'ctcgaattcggnaarathxgngt;

JH2s 5'ctcgaattcgadggnatgtggaayathac;

JH2a 5'acactcgagtgnacngtdatrttccacat;

JH3a 5'acactcgagcatraangcnggngcrtacat;

JH4s 5'ctcgaattcaratgacngtngcnttygc;

JH5s 5'ctcgaattcggnathtggtadtggtt;

JH5a 5'acactcgagaaccartaccadatncc;

JH6a$_1$ 5'acactcgagggptcraarcanggrtt;

JH6a$_2$ 5'acactcgagccrtadatntrnggrtc.

PCR reactions were set up in a dedicated fume hood using aerosol resistant filter tips (Quality Scientific Plastics), to prevent contamination. 1 µg of *X. laevis* genomic DNA was amplified in a Perkin-Elmer-Cetus thermal cycler in 50 µl containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM dNTP's, 250 ng of the degenerate primers and 2.5U Taq DNA polymerase (Gibco BRL). PCR amplifications were performed as follows: denaturation at 93^{0}C for 2.5 minutes, followed by 35 cycles of 93^{0}C for 1 minute, 55^{0}C for 2 minutes and 72^{0}C for 3 minutes, and ending with an extension step at 72^{0}C for 5 minutes. 10 µl of the reaction products were electrophoresed through a 2% MetaPhor (FMC) TBE agarose gel containing ethidium bromide (50 ng/ml).

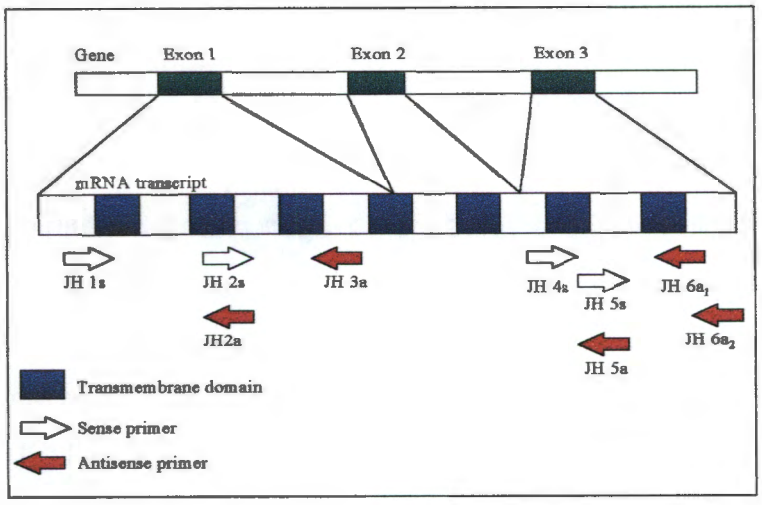


Figure 2.1. Schematic diagram of the location of the degenerate primers relative to the mouse GnRH receptor gene and cDNA. Diagram is not to scale.

2.3.4 Southern Blot Analysis

PCR products were electrophoresed through a 1% agarose TBE gel. The DNA in the gel was denatured for 30 minutes in denaturing solution (1.5 M sodium chloride; 0.5 M sodium hydroxide), and then neutralised by washing twice in neutralising solution (1.5 M sodium chloride; 0.5 M Tris-HCl, pH7.2; 1 mM EDTA) for 15 minutes. DNA was transferred to nitrocellulose membrane (Hybond N$^+$, Amersham) overnight in 20 x SSC (3 M NaCl; 0.3 M sodium citrate, pH7.2) by capillary blot. The membrane was

washed in 2 x SSC, and fixed for 20 minutes in 0.4 M sodium hydroxide. Probes were labelled to a specific activity of approximately 7 x 10^8 cpm/μg using [α^{32}P]dCTP (3000Ci/mmol) and the Megaprime labelling kit (both Amersham). Labelled probes were purified from unincorporated deoxynucleotides using a Sephadex G-50 spin column, equilibrated with 100 μl of STE (10 mM tris-HCl, pH 8; 1 mM EDTA; 100 mM NaCl). Probes used are specified in results. Blots were prehybridised, for 1 h prior to an overnight hybridisation in hybridisation solution containing the denatured [^{32}P]-labelled probe, 50% deionised formamide, 100 μg/ml denatured herring sperm DNA, 2 x Pipes (0.02 M piperazine-N,N'-bis[2-ethanesulphonic acid, 0.8 M NaCl) and 0.5% SDS. Membranes were washed for 15 minutes at room temp in 1 x SSC, 0.1% SDS followed by a 20 minutes wash at 50°C in 0.5 x SSC, 0.1% SDS, and a second 20 minutes wash at 50°C in 0.2 x SSC; 0.1% SDS. Membranes were subjected to autoradiography and in most cases a 3 to 5 h exposure at -70°C was sufficient to see results.

2.3.5 PCR product subcloning and analysis

PCR products of interest were excised from low melting point agarose gels (FMC BioProducts) and subcloned using Amersham pMOS*blue* T-vector kit (see appendix 7.1 for map), according to manufacturer's specifications. Recombinant colonies were screened for inserts using either colony hybridisation to a probe of interest or colony PCR. For colony hybridisation colonies were grided onto agar plates containing ampicillin (100 μg/ml) in duplicate. Colonies were lifted onto Hybond N^+ nitrocellulose (Amersham) 72 mm disc membranes in duplicate, denatured in denaturing solution (1.5 M sodium chloride; 0.5 M sodium hydroxide) for 7 minutes, neutralised twice in neutralising solution (1.5 M sodium chloride; 0.5 M tris-HCL, pH7.2; 1 mM EDTA) for 3 minutes, fixed for 20 minutes in 0.4 M NaOH and probed as for Southern blots. For the purposes of colony PCR, colonies of interest were picked in 20 μl of sterile water. 15 μl was used to inoculate an overnight culture, while the other 5 μl was used for PCR amplification in 20 μl with 1.5 mM $MgCl_2$, 200 mM dNTPs, 20 mM Tris-HCL (pH8.4), 50 mM KCL, 10 pmoles of vector specific primers (see appendix, for vector maps), 1 U of Taq DNA polymerase (Gibco BRL). PCR cycle conditions were set up as for the degenerate primer PCR (2.3.3). PCR products were analysed by agarose gel electrophoresis followed by Southern blotting

or direct SDS-alkaline lysis mini-preparation of DNA from positive colonies. DNA of interest was sequenced (T7 Sequenase version 2.0, Amersham).

2.3.6 Screening and analysis of the Genomic DNA library

A *X. laevis* Lambda EMBL4 (see appendix 7.2 for map) genomic library was kindly supplied by Dr T. Sargent (NIH). The library was made from DNA extracted form red blood cells of a female homozygous diploid frog line (HD-1) of *X. laevis*, prepared by gynogenesis by Dr Robert Tomkins. The DNA was subjected to a partial digestion with Sau3A and the 15-20 kbp fraction was recovered from a sucrose gradient. The DNA was ligated to the EMBL4 BamH1-Sal1 cut vector, packaged *in vitro* and plated. The titer of the library was established as 1.6×10^9 pfu/ml, by plating on *Escherichia coli* LE392 [Leder *et al.*, 1977]. A million plaques were screened by filter hybridisation with Hybond N^+ (Amersham), as described by Benton and Davis (1977). PCR generated subclone p*Xl*a.1 (see appendix 7.3) was used as a probe, and was labelled as described above, using the Amersham Megaprime labelling kit. Primers JH5s and $JH6a_2$ were substituted for the random hexamers, due to the low specific actvity generated with the random primers. Phage DNA from positive plaques, which were subjected to secondary and tertiary screenings, was prepared using the small scale liquid lysate method [Benson and Taylor, 1984]. DNA was analysed by appropriate restriction endonuclease digestion, followed by Southern blotting. The genomic clone EcoR1 restriction endonuclease fragment of approximately 3 kbp was subcloned into the dephosphorylated EcoR1 site of pBSK(+/-) (Stratagene, see appendices 7.4 and 7.5 for maps), and sequenced with the T7 Sequenase system (Amersham), using gene specific primers.

2.3.7 Amplification of cDNA

Several approaches were used for attempted PCR amplification of full-length cDNA, using gene specific primers designed from the subclones obtained after PCR on genomic DNA (p*Xl*a.1) and sequencing of the genomic clone (p*Xl*a.2). See appendices 7.3 and 7.5b for the primer design and location. These approaches were mainly adapted from the initial RACE (rapid amplification of cDNA ends) method described by Frohman *et al.* (1988). This method was used, but no conclusive results were obtained. An adaptation of this method, described by Chenchik *et al.*(1995) was finally used to successfully clone the full-length cDNA of the pituitary

GnRH receptor. This method made use of a Marathon cDNA kit (Clonetech), which requires the ligation of adapter primers at the 5' ends of double stranded cDNA which is more efficient than homopolymeric tailing described by Frohman *et al.* (1988). 5 μg of total RNA was digested with 5 U of RNase free DNase I for 30 minutes at 37^{0}C, followed by extraction with phenol to remove the enzyme, and precipitation with 100% ethanol. cDNA synthesis from 1μg of total RNA, was performed according to kit specifications with [α-^{32}P]dCTP (1 μCi/μl, Amersham), in order to facilitate quantification of cDNA synthesis. Double stranded cDNA was analysed by agarose gel electrophoresis followed by autoradiography. Adaptor primers were ligated onto the 5' ends of the double stranded cDNA. The cDNA was then amplified in a thermal cycler (Perkin-Elmer) in 50 μl containing 200 mM dNTPs, adaptor primers and gene specific primers (*Xl*1a.1a and *Xl*1a.2a, see appendix 7.5b) at 10 μM and 1x Advantage KlenTaq polymerase mix (Clonetech) with its reaction buffer. PCR amplifications were performed as follows: denaturation at 94°C for 1 minutes, followed by 35 cycles of denaturation at 94°C for 30 sec. and either annealing at 64°C for 30 sec. and extension for 3 minutes at 68°C or annealing and extension for 3 minutes at 68°C. Negative controls for the PCR were as follows: no cDNA, and an adaptor only control. This control is used to determine if any of the products generated are as a result of amplification with only the adaptor primer. In some of the amplifications a negative control where only gene specific primers were added, was included, in order to determine if any products were a result of non-specific amplification. A positive control cDNA, with gene specific primers was included in the kit. After two rounds of PCR, using nested gene specific and adaptor primers, products of all the reactions were loaded onto a 1% agarose gel and subjected to Southern blotting. 100 pmoles of oligonucleotide probe (*Xl*1a.4s, see appendix 7.5b) was labelled with [γ^{32}P]dATP at 5000 Ci/mmol and 10 U of T4 polynucleotide kinase (both Amersham) for 1 h at 37°C. Following the labelling reaction, the probe was purified on a Sephadex G-10 spin column equilibrated with 100μl of 1 x STE (10 mM tris-HCl, pH 8; 1 mM EDTA; 100 mM NaCl). The membrane was prehybridised for 2 h prior to an overnight hybridisation in hybridisation solution consisting of; the labelled oligonucleotide probe in 5 x Denhardts reagent (0.1% (w/v) of Ficoll, polyvinylpyrrolidone, and BSA); 6 x SSC (0.9 M NaCl. 0.09 M sodium citrate, pH 7.2)) and 0.5% (w/v) sodium dodecyl sulphate (SDS). The hybridisation temperature was 5°C lower than the melting temperature of the oligonucleotide, which was calculated

from the nucleotide sequence as, 2(A+T)+4(G+C). After hybridisation, the membrane was subjected to low stringency washing for 5 minutes at room temperature in 5 x SSC, and 0.1% SDS, followed by autoradiography. Any positive products were analysed as in section 2.3.5 (*PCR product subcloning and analysis*).

2.3.8 Isolation of a full-length cDNA clone

The 1.2 kbp cDNA encoding the full-length receptor was amplified from Marathon pituitary cDNA using Pfu DNA polymerase (Stratagene) and primers designed to the 5' and 3' untranslated regions. The 5' sense primer was designed to the untranslated region of the sequence of the marathon clones and the 3' antisense primer was designed to the untranslated region of p*Xl*a.2 (see appendix 7.5 and fig. 2.6, primer *Xl*1a.1a). Five individual cDNA clones were sequenced in both the forward and reverse directions (T7 sequenase version 2.0, Amersham). Where sequences varied (see section 2.4.3) the relevant region of the genomic clone, amplified by PCR was sequenced for verification.

2.3.9 Cell Culture

COS-1 cells were grown in 25 ml cell culture flasks (Corning) in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat inactivated foetal calf serum in a 5% CO_2 incubator. Cells were subcultured when 70% confluent using 0.25% trypsin, 0.04% EDTA in PBS (137 mM NaCl, 2.68 mM KCl, 0.43 mM NaH_2PO_4, 1.47 mM KH_2PO_4, pH 7.4). Cells were maintained in antibiotic-free medium.

2.3.10 Transient transfection of COS-1 cells

The *X. laevis* GnRH receptor cloned from pituitary cDNA was subcloned into the BamHI and XhoI sites of pcDNA 1/Amp (Invitrogen) (see appendix 7.6), expression vector containing the SV40 early promoter. DNA for transfection was prepared from a 500 ml overnight culture of 2 x YT Broth (10 g/L NaCl, 10 g/L Yeast extract, 16 g/L Bactotryptone) containing Ampicillin (100 μg/ml). Plasmid DNA was extracted using the Wizard DNA purification system (Promega). 24 h prior to transfection, cells were plated at appropriate density on poly-*D*-lysine coated dishes, in DMEM containing 10% foetal calf serum, penicillin (0.2 U/ml) and streptomycin sulphate (100 μg/ml) (Gibco BRL). DNA was transfected in serum-free DMEM for 4 h using an adapted DEAE-Dextran method [Keown *et al.*, 1990], with a 50 minute chloroquine (200 μM)

treatment and a 2 minute 10% dimethylsulfoxide shock [Luthman and Magnusson, 1983; Lopata *et al.*, 1984]. For studies on membrane receptor binding 3 x 10^6 cells/10 cm dish (Corning) were transfected with 15 μg of DNA. For assays of inositol phosphate production and whole cell binding assays, 2 x 10^5 cells/well (12 well plates, Corning) were transfected with 2μg of DNA. Cells were grown for 48 h after transfection, before each experiment.

2.3.11 Receptor Binding Assay

Both cell membrane assays and whole cell binding assays were tested. For membrane binding, cell membranes were prepared from transfected cells, as described in Millar *et al.* (1989). The receptor binding assay was performed using 80000 cpm of ^{125}I-[D-Ala^6, N-Me-Leu^7,Pro^9-NHEt]GnRH, ^{125}I-[Ant26]GnRH (Antagonist 26) and ^{125}I-[His^5, D-Arg^6, Trp^7, Tyr^8]GnRH, which were radioiodinated according to an adapted chloramine-T method [Millar *et al.*, 1995]. For whole cell binding, cells were washed twice for 5 minutes at 4°C in buffer I (140 mM NaCl; 4 mM KCl; 20 mM HEPES; 8.6 mM glucose; 1 mM $CaCl_2$; 1 mM $MgCl_2$; 0.1% BSA, fatty acid free; pH7.4), followed by incubation for 3 h at 4°C with 100 000 cpm of labelled GnRH agonist and an appropriate amount of unlabelled GnRH agonist. The binding at 10^{-6} mGnRH agonist was considered to represent the non-specific binding (NSB). After incubation the cells were washed three times in buffer I to remove unlabelled peptides, removed from the plates with 0.5 M NaOH and bound radioactivity counted.

2.3.12 Inositol Phosphate Assay

Transfected COS-1 cells were labelled overnight in 0.5 ml of Medium 199 (Gibco BRL), containing 2% foetal calf serum and 2 μCi/ml myo[2-^{3}H]inositol (Amersham) as described in Millar *et al.*, (1995). Cells were washed twice for 5 minutes at 37°C in buffer I, followed by stimulation with GnRH, as required, in buffer I and 10 mM LiCl for 1 h at 37°C, with gentle agitation. Inositol phosphates were extracted with 10 mM formic acid for a minimum of 30 minutes at 4°C, as described in Berg *et al.*, (1994). Total inositol phosphates (IP1, IP2 and IP3) were separated on a Dowex ion exchange column, eluted with 3 ml of 1 M ammonium formate/0.1 M formic acid and radioactivity determined. Data points were determined in duplicate, and data

reduction was determined using GRAPHPAD PRISM. ED_{50} values are the mean of three separate experiments.

2.4 Results

2.4.1 A putative GnRH receptor amplified from genomic DNA

After amplification of genomic DNA using the degenerate primers, products were blotted onto nitrocellulose and probed with mouse GnRH receptor cDNA. A positive band of approximately 120 bp was obtained with the primer pair, JH5s and JH6a2 (fig. 2.1). The other primer pairs, gave products of the correct size on ethidium bromide stained agarose gels, but these products did not hybridise to the mouse cDNA probe (fig. 2.2). The positive 120 bp band was subcloned and sequenced. Of the ten clones analysed, two different subtypes were identified (clone *Xl*a.1 and clone *Xl*b.1), which showed homology to the mammalian GnRH receptors (see appendix 7.3 and 7.7 for sequences). Clone *Xl*b.1 will be discussed in greater detail in chapter 3.

2.4.2 Genomic clone for pXla.1 receptor subtype isolated

Clone p*Xl*a.1 was used to screen a *Xenopus laevis* genomic DNA library. DNA from eight positive plaques was extracted, after secondary and tertiary screening. Restriction endonuclease digestion and Southern blot analysis showed that the genomic clones are approximately 14-16 kbp (fig. 2.3). Digestion with EcoRI and SalI restriction endonucleases followed by Southern blotting revealed a 3 kbp fragment which hybridised to p*Xl*a.1. Subcloning this 3 kbp EcoR1 fragment (fig. 2.3, see appendices 7.5a and b) of the genomic clone yielded the sequence of this entire exon, consisting of transmembrane domains 6 and 7, as well as an intracellular carboxy-terminal tail.

2.4.3 Full-length cDNA of pXla isolated from pituitary RNA

In order to amplify the GnRH receptor from the pituitary, cDNA with adaptors ligated onto the 5' ends, was synthesised using the Marathon cDNA synthesis kit (Clonetech). The quality of the pituitary cDNA constructed using the Marathon cDNA kit (Clonetech) was assessed in comparison to control cDNA (supplied by kit) by agarose gel electrophoresis followed by autoradiography (fig. 2.4). The pituitary

cDNA was then amplified in two rounds of nested PCR, using gene specific primers designed to p*Xl*1a.2 (see appendix 7.5a and b), followed by Southern blot analysis, three specific products of 10 kbp, 1.2 kbp and 0.8 kbp were amplified by PCR with an annealing temperature of 64°C (fig. 2.5). Only the 800 bp band was amplified with an annealing temperature of 68°C (fig. 2.5). The cDNA encoding the full-length receptor was amplified using primers designed to the 5' and 3' untranslated regions. The 1.2 kbp band was subcloned into the pMOS*Blue*-T vector and sequenced using gene specific primers. Figure 2.6 shows the nucleotide and deduced amino acid sequence of the cDNA and the location of primers used for nested PCR and sequencing. Five different cDNA clones were sequenced and despite the use of the high fidelity polymerase, Klentaq, (Clonetech) and Pfu DNA Polymerase (Stratagene) [Barnes, 1994] differences between the five clones were present. Three amino acid mutations were present: G to an A in transmembrane domain (TM) I, resulting in Ala^{55} to Thr change was present in two of the clones, in TM V an A to a T mutation resulting in Tyr^{211} becoming a Phe in one of the clones and a T to G mutation causing a stop codon at Tyr^{238} in TM IV was also present in only one of the clones. Because none the five clones contained all three nucleotide changes, the consensus sequence was deduced to be the correct sequence, and was confirmed by sequencing of the relevant area of the genomic clones.

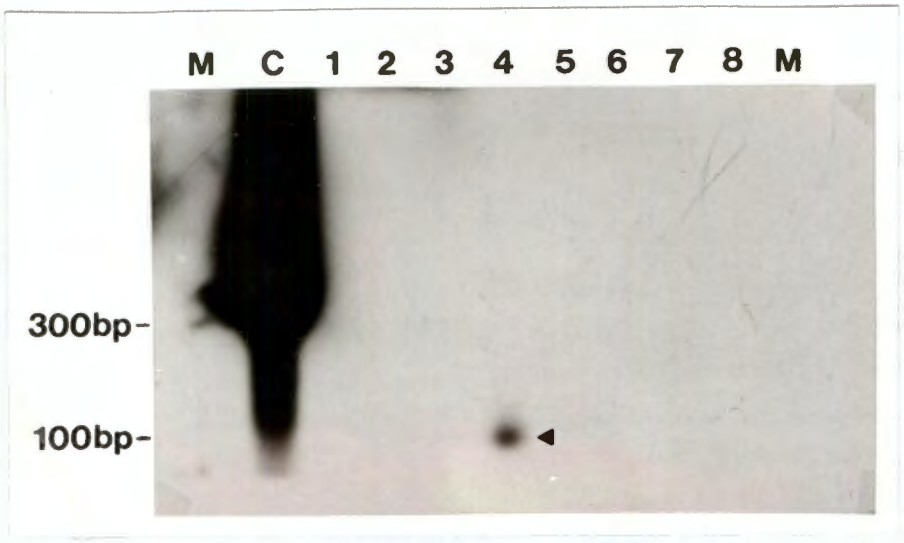


Figure 2.2. Southern blot of the products after PCR on *X. laevis* genomic DNA with the degenerate primers, probed with the mouse GnRH receptor cDNA. M represents the MW marker, C, mouse cDNA as the positive control and 1-8, the combinations of degenerate primers used. 1: JH1s/JH2a; 2: JH4s/JH6a_2; 3: JH5s/JH5a_2; 4: JH5s/JH6a_1; 5: JH4s/JH6a_1; 6: Primer blank. A 120 bp product which hybridises to the mouse receptor cDNA could be seen with the primer pair; JH5s and JH6a_2.

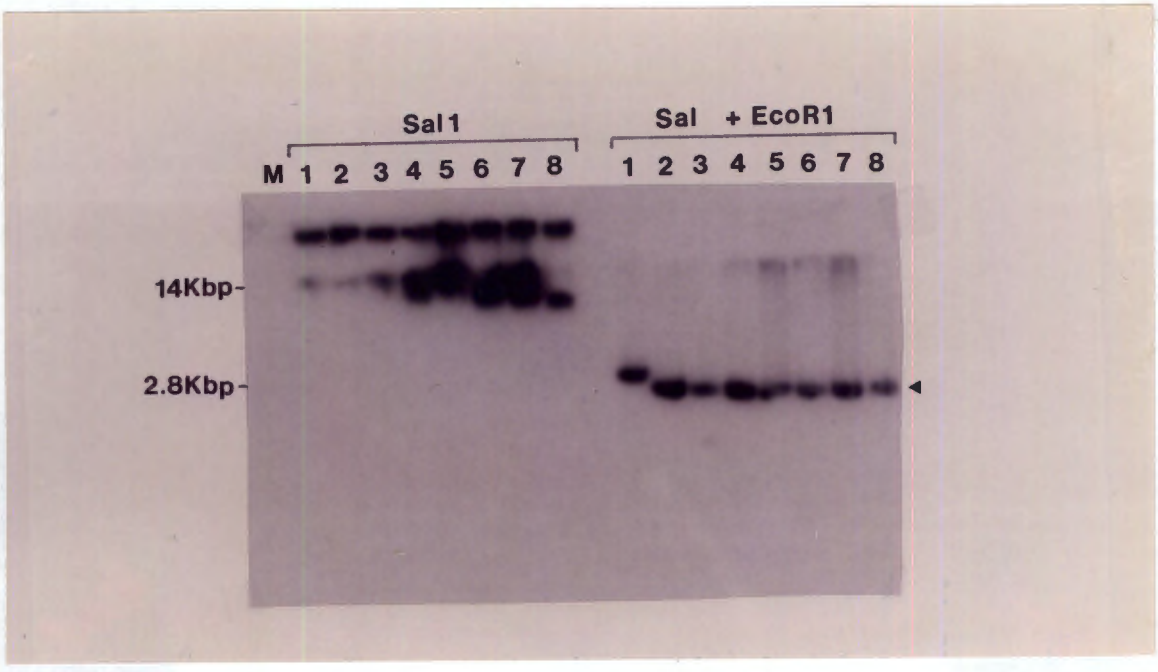


Figure2.3. Southern blot probed with labelled p*Xl*a.1, of the 8 genomic clones (1-8) isolated after the tertiary screening of the *X. laevis* genomic DNA library with *Xl*1a. The clones are digested with Sal1 and Sal1 plus EcoR1. Sal1 will excise the insert from EMBL4. The 3 kbp band, which hybridised to the probe was subcloned into pBluescript (Stratagene, see appendix 7.4), and recombinant clones sequenced (se appendices 7.5a and b). M represents the MW marker.

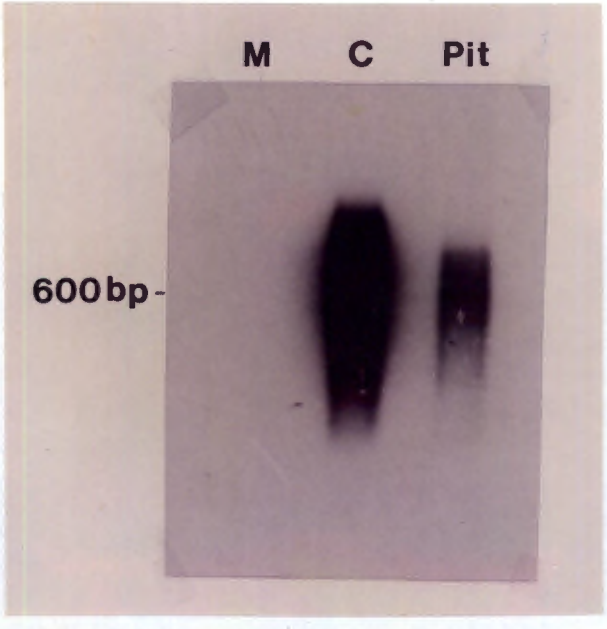


Figure2.4. Autoradiograph showing the *X. laevis* pituitary cDNA after the first strand synthesis (Pit). A positive control was provided with the Marathon cDNA amplification kit (Clonetech) (C). Most of the cDNA was approximately 600 bp. M represents the MW marker.

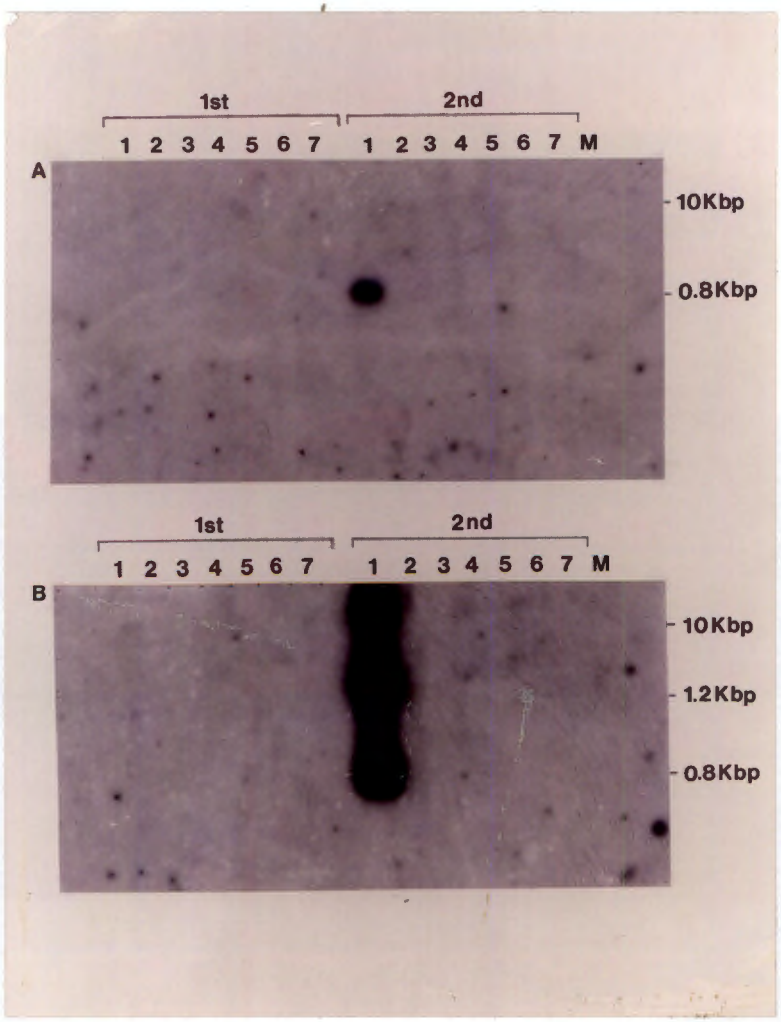


Figure 2.5. Southern blot after two rounds of nested Marathon PCR on *X. laevis* pituitary cDNA. The first (1st) and second (2nd) nested PCR products were probed with a labelled nested primer. A 0.8 kbp product could be seen after amplification with an annealing temperature of 64°C (**A**), while 10 kbp, 1.2 kbp and 0.8 kbp products could be seen after PCR with an annealing temperature of 68°C (**B**). The 1.2 kbp product was subcloned and sequenced revealing the 5' sequence of the *X. laevis* GnRH receptor. 1: *X. laevis* pituitary (Pit.) cDNA amplified with adaptor primers (AP) and frog gene specific primers; 2: positive control cDNA; 3: Pit. cDNA with only gene specific primers; 4: Pit. cDNA with only adaptor primers; 5: positive control cDNA with only gene specific primers, 6: positive control cDNA with only adaptor primers; 7: no DNA negative control.

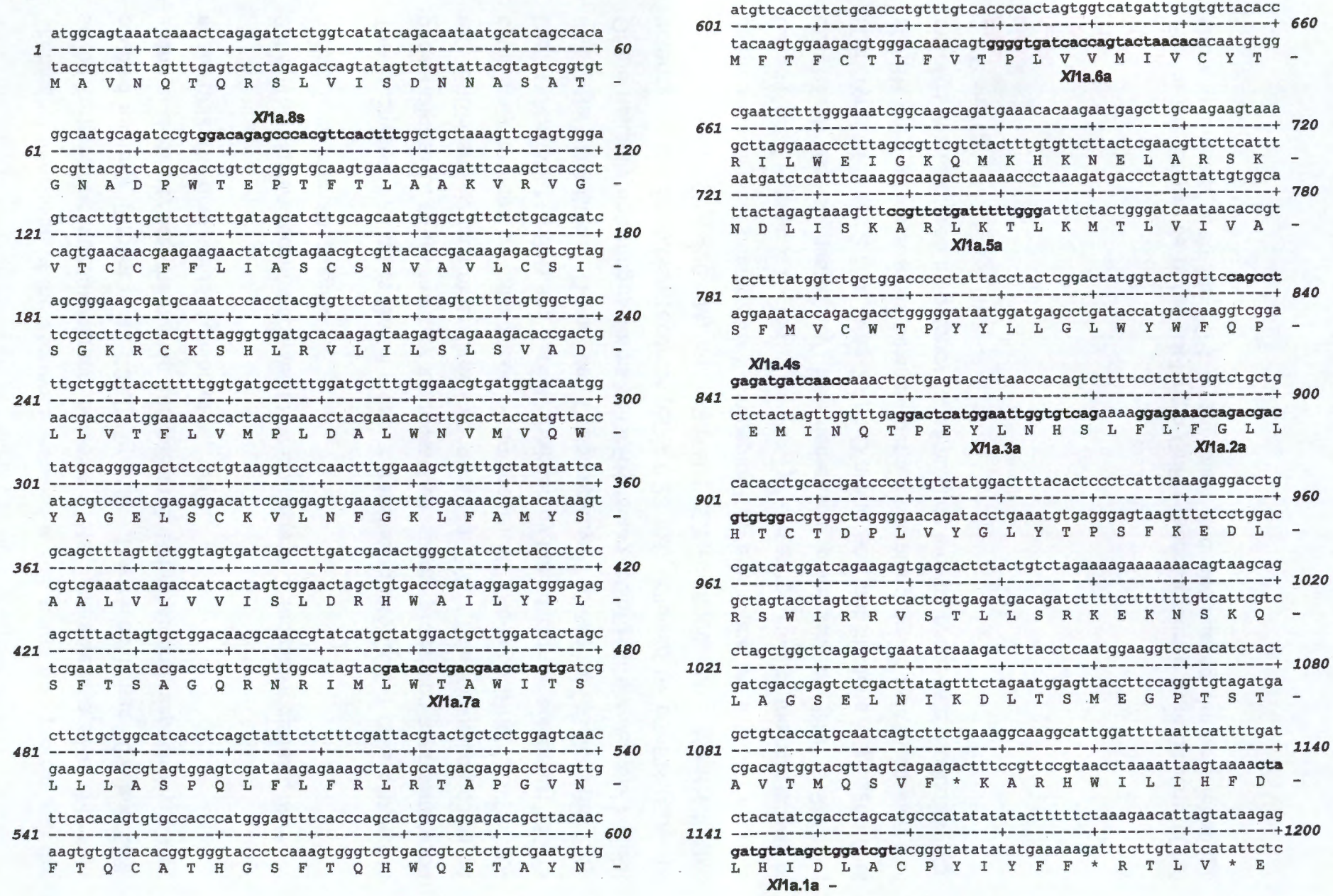
Theoretical translation of the sequence revealed a 368 amino acid protein with the seven hydrophobic transmembrane domains (TMs), characteristic of a G-protein coupled receptor. [Probst *et al.*, 1992] (fig. 2.6). This receptor has 46% and 55% amino acid identity with the human and catfish GnRH receptors, respectively [Kakar *et al.*, 1992; Chi *et al.*, 1993; Tensen *et al.*, 1997].

2.4.4 The pituitary receptor couples to phospholipase C and distinguishes GnRH natural ligands and analogues

The recombinant *X. laevis* GnRH receptor transfected into COS-1 cells coupled to phospholipase C, as revealed by the inositol phosphate production after stimulation with GnRH and various other GnRH agonists (fig. 2.7). This stimulation could be completely inhibited by the addition of antagonist 26 ([Ac-D-p-ClPhe^{1,2}, D-Trp^{3}, D-Lys^{6}, D-Ala^{10}NH$_2$] GnRH) and partially inhibited by the addition of antagonist 135-18 ([Ac-D-Nal^{1}, D-4-ClPhe^{2}, D-Pal^{3}, Ile^{5}, D-IsopropylLys^{6,8}, D-Ala^{10}NH$_2$]GnRH) (fig. 2.8). Of the naturally occurring isoforms of GnRH tested, cGnRH II showed the highest potency for the amphibian receptor (ED_{50} 0.23 nM), followed by sGnRH (ED_{50} 18 nM), mGnRH (ED_{50} 420 nM) and cGnRH I (ED_{50} >10000 nM). cGnRH II also showed the highest potency for the receptor than any of the other GnRH agonists tested (fig.2.7 A, table 2.1). His^{5}, Trp^{7} and Tyr^{8} are all required for the high activity of cGnRH II, as suggested by the decreased potencies of [His^{5}]GnRH and [Trp^{7},Tyr^{8}]GnRH (fig.2.7 B, table 2.1). D-amino acid substitutions in position 6 of cGnRH II do not increase its potency, but a constrained amino acid in position 6 of mGnRH does increase its potency for stimulating inositol phosphate production (fig. 2.7 B and C).

Figure 2.6. Sequence of the full-length GnRH receptor cloned from pituitary cDNA showing primers used for PCR amplification and sequencing (in bold lettering).

Figure 2.6.



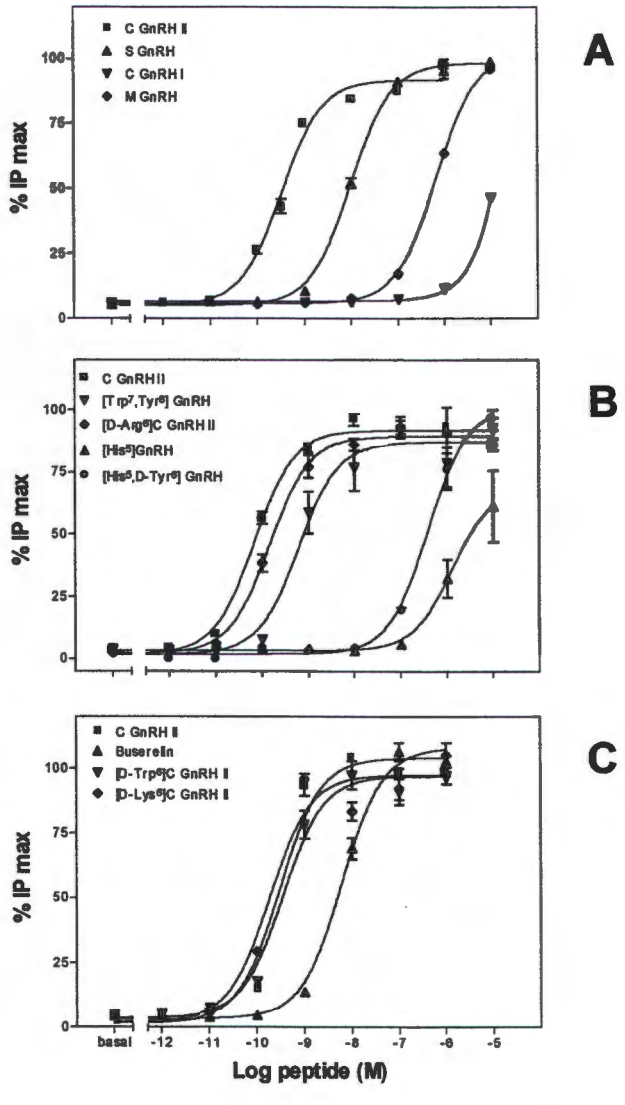


Figure 2.7. Inositol phosphate production in COS-1 cells transfected with the *X. laevis* pituitary GnRH receptor and stimulated with **A:** Chicken GnRH II (■), Salmon GnRH (▲) mammalian GnRH (◆), and chicken GnRH I (▼); **B:** Chicken GnRH II (■), [Trp^7, Tyr^8]GnRH (▼), [His^5, D-Arg^6, Trp^7, Tyr^8]GnRH (◆), [His^5]GnRH (▲), [His^5, D-Tyr^6]GnRH (●); and **C:** Chicken GnRH II (■), Buserelin (▲), [His^5, D-Trp^6, Trp^7, Tyr^8]GnRH (▼), and [His^5, D-Lys^6, Trp^7, Tyr^8]GnRH (◆). Data represent duplicates from a representative experiment.

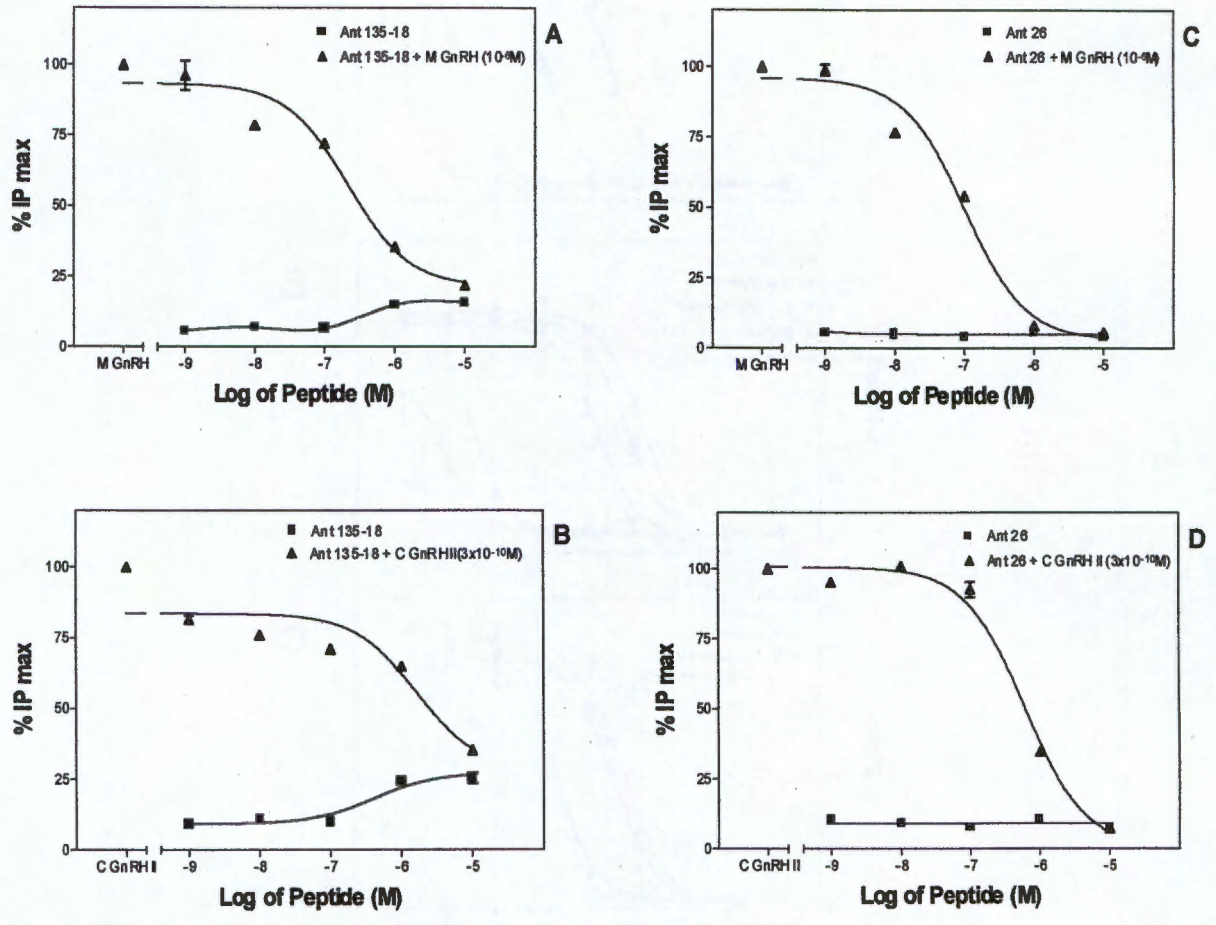


Figure2.8: *Inhibition of inositol phosphate production by GnRH antagonists.*
Partial inhibition of stimulation with mammalian GnRH (10^{-6} M) (**A**) and chicken GnRH II ($3x10^{-10}$ M) (**B**) by antagonist 135-18 ([Ac-D-Nal[1], D-4-ClPhe[2], D-Pal[3], Ile[5], D-IsopropylLys[6,8], D-Ala[10]NH_2]GnRH). Inhibition of stimulation with mammalian GnRH (10^{-6} M) (**C**), and chicken GnRH II ($3x10^{-10}$ M) (**D**) by antagonist 26 ([Ac-D-p-ClPhe[1,2], D-Trp[3], D-Lys[6], D-Ala[10]NH_2] GnRH). The mammalian receptor antagonist 135-18 is a partial agonist to the *Xl*a receptor, while antagonist 26 in an antagonist in both the mammalian and the frog receptors.

Table 2.2. ED_{50}s of GnRH agonists for inositol phosphate production in COS-1 cells transfected with the *X. laevis* pituitary GnRH receptor. ED_{50} values are the mean of three separate experiments. Mean = SEM, data reduction was performed using Graphpad Prism

ED_{50} (nM)	Peptide
420 ± 100	mammalian GnRH
0.23 ± 0.1	[His^5, Trp^7, Tyr^8] GnRH (chicken GnRH II)
18 ± 10	[Trp^7, Leu^8] GnRH (salmon GnRH)
>10000	[Gln^8] GnRH (chicken GnRH I)
6.6 ± 0.1	[T-BuSer^6, Pro^{9}NHEt] GnRH (Buserelin)
1095 ± 300	[His^5]GnRH
5 ± 3	[Trp^7,Tyr^8]GnRH
400 ± 15	[His^5, D-Tyr^6]GnRH
0.2 ± 0.1	[His^5, D-Arg^6, Trp^7 Tyr^8] GnRH
0.47 ± 0.2	[His^5,D-Trp^6, Trp^7,Tyr^8]GnRH
0.3 ± 0.14	[His^5,D-Lys^6, Trp^7,Tyr^8]GnRH

2.4.5 Binding of GnRH analogues to the pituitary receptor could not be detected

Both whole cell and membrane binding assays were performed, but no specific binding with ^{125}I labelled [D-Ala^6,NMe-Leu^7,Pro^9-NHEt]GnRH, [D-Arg^6]cGnRH II and antagonist 26 could be detected. This might indicate that the *X. laevis* receptor has a low expression in the transiently transfected COS-1 cells or has a relatively low affinity for the ligands tested.

2.4.6 The X. laevis pituitary GnRH receptor gene consists of three exons.

Gene specific primers were used to amplify regions of the gene in order to determine the location of the introns within the gene (fig. 2.9). It contains two introns within the coding region, intron II and III. Intron II is approximately 4.5 kbp, while intron III is approximately 6.8 kbp. There is a third intron (intron I) three nucleotides, prior to the start codon, in the 5' untranslated region, of approximately 1.3 kbp.

2.5 Discussion

PCR amplification of genomic DNA revealed a short fragment of a putative receptor with homology to the mammalian GnRH receptors. This was used to identify a genomic clone from a *X. laevis* genomic DNA library. The full-length cDNA of this receptor was cloned from the pituitary and revealed a 368 amino acid receptor. This receptor has a 46% amino acid identity with the human GnRH receptor [Kakar *et al.*,

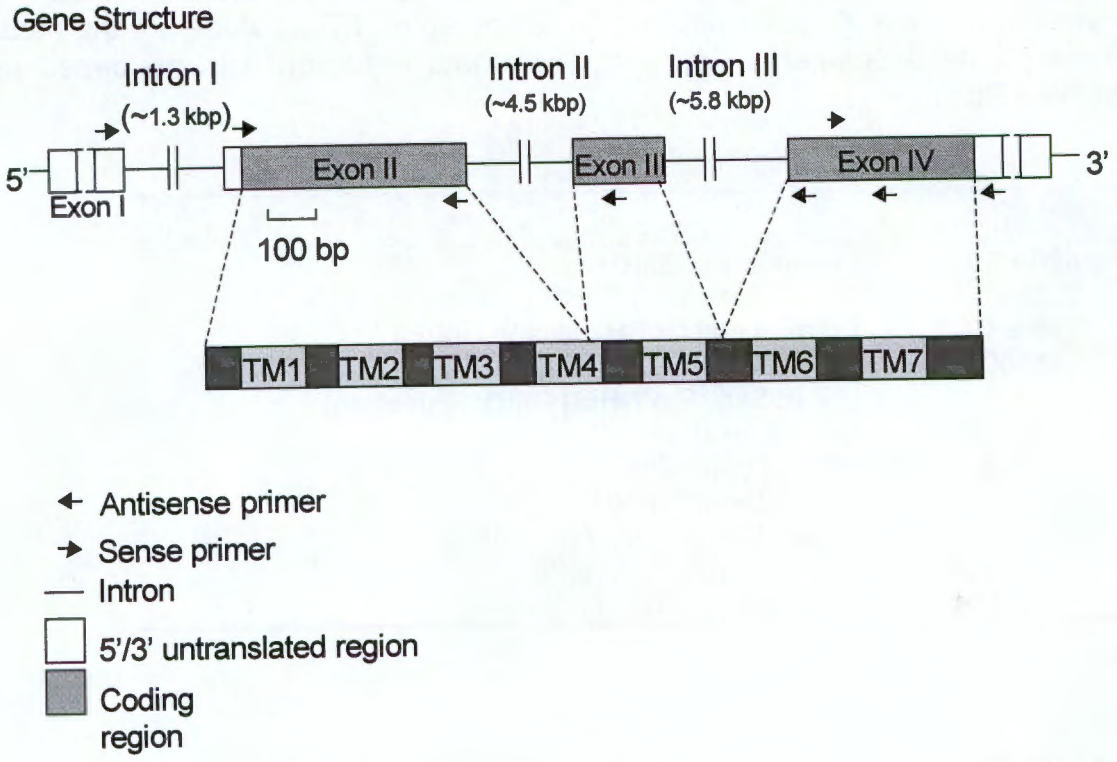


Figure 2.9. Schematic representation of the *X. laevis* GnRH receptor gene showing the three exons and two introns. The entire gene is between 12 and 13 kbp. The location of the primers used for gene analysis and sequencing are shown.

1992; Chi *et al.*, 1993], and a 55% amino acid identity with the cloned catfish GnRH receptor [Tensen *et al.*, 1997].

The amino terminus is very poorly conserved with the human GnRH receptor (fig. 2.10) and the catfish GnRH receptor. The glycosylation site Asn^{18} in the human receptor is not conserved, but there is an N-linked glycosylation consensus site at Asn^{16}. The cysteines, Cys^{14} in the amino-terminus of the human GnRH receptor and Cys^{200} in extracellular loop II, which have been suggested to form a disulphide bridge [Davidson *et al.*, 1997] are not conserved. Cook and Eidne [1997], however, have found that this disulphide bridge is not important for mammalian GnRH activity in the rat receptor. The two cysteines in extracellular loops I and II, which have been shown for the mammalian GnRH receptors [Davidson *et al.*, 1997; Cook and Eidne, 1997], as well as other GPCRs [for review, see Strader *et al.*, 1994], to form a

disulphide bridge, Cys^{107} and Cys^{184} (Cys^{114} and Cys^{196} in the human GnRH receptor), are, however, conserved in the *X. laevis* pituitary receptor.

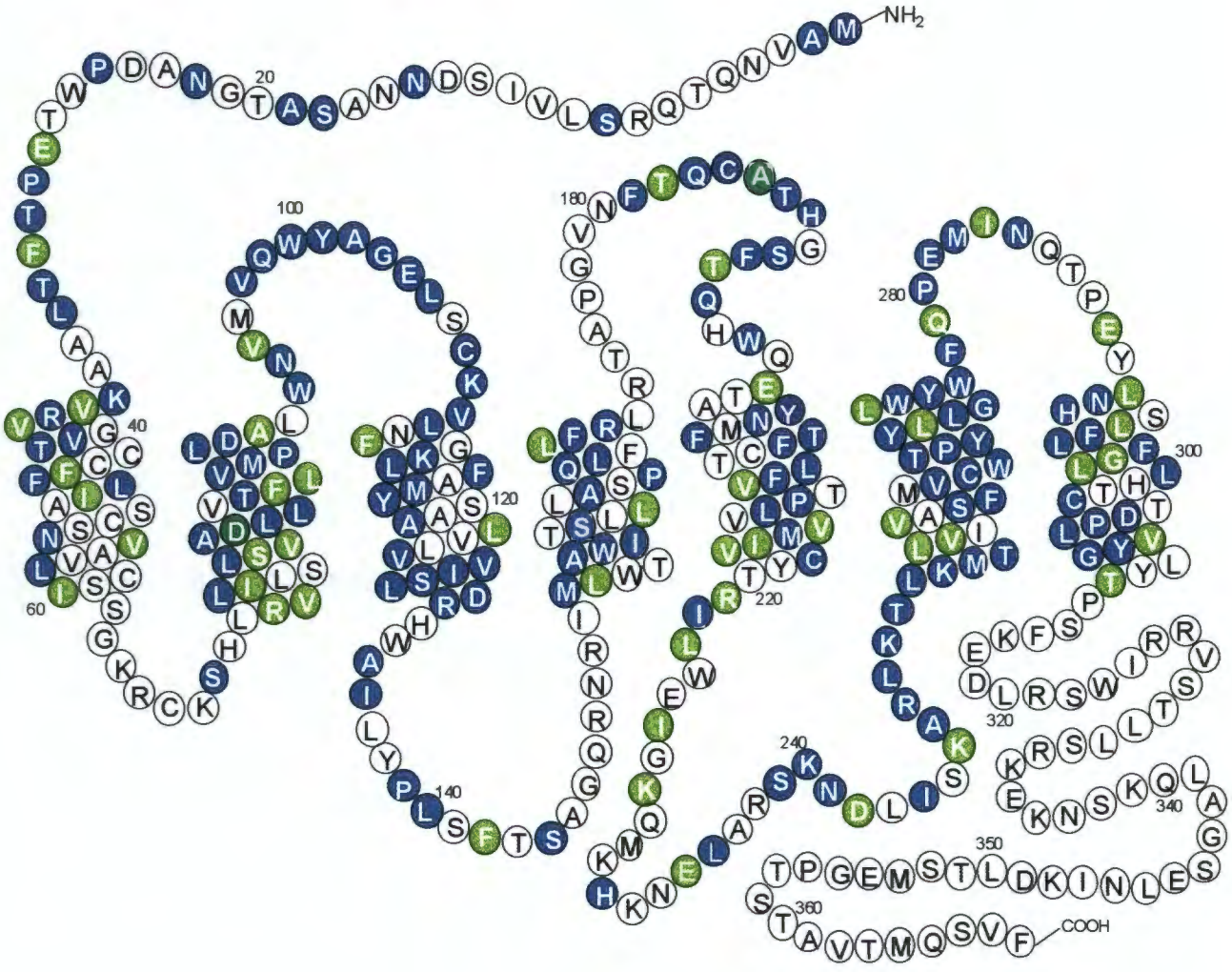


Figure 2.10. Diagram of the *Xenopus laevis* pituitary GnRH receptor, showing amino acid residues, which are conserved with the human GnRH receptor (blue) and conservative substitutions (green).

The transmembrane domains (TM) of the *X. laevis* receptor are highly conserved with the human GnRH receptor (fig. 2.10). TM VI and III are the most conserved, at 69 and 62% identity respectively, followed by TMs VII, IV, II, V, and I. The high

conservation seen in the TM helices, is required for the formation of the hydrophobic α-helices, which anchor the receptor in the membrane and for helix/helix interactions through specific amino acid side chains. In general, the TM domains are highly conserved among GPCRs [for review, see Strader *et al.*, 1994]. The high conservation within receptor types, in these domains, may also reflect amino acid residues, which are important for ligand binding. All the amino acid residues in the TMs of the mammalian GnRH receptors, which have been shown to be important for ligand interactions are conserved in the amphibian receptor [for review, see Sealfon *et al.*, 1997]. Asp^{91} in the frog GnRH receptor, which aligns to Asp^{98} in TM II of human receptor and proposed to interact with His^{2} of GnRH, and Lys^{114} of the frog receptor which aligns to Lys^{121} in TM III of the human GnRH receptor, which may interact with His^{2} or $pGlu^{1}$ of GnRH [Zhou *et al.*, 1995; Rodic *et al.*, 1996; for review, see Sealfon *et al.*, 1997]. Asn^{102} in TM II, which interacts with the glycine amide at the carboxy-terminus of GnRH is also conserved (Asn^{95}, in the *X. laevis* receptor) [Davidson *et al.*, 1996(b)].

Asn^{87} in the human GnRH receptor has been shown by mutagenesis, to interact with Asp^{318} in TM VII [Zhou *et al.*, 1994]. Mutagenesis of Asn^{87} to an Asp in the human receptor was inactive, unless a second mutation of Asp^{318} to a Asn was also incorporated [Zhou *et al.*, 1994]. Two aspartic acid residues in these positions in TMs II and VII of the human GnRH receptor, therefore, affected the receptor in such a way as to render it inactive. Interestingly Asn^{87} in TM II of the human GnRH receptor is an Asp in the *X. laevis* receptor (Asp^{80}). The *X. laevis* receptor, therefore, has an aspartic acid in both TMs II and VII (Asp^{80} and Asp^{305}). This arrangement is presumably functional in the *X. laevis* receptor due to other co-ordinated amino acid changes, which have yet to be identified. Mutations of the catfish GnRH receptor have revealed that the aspartic acid residue in TM II (Asp^{90}) is important for the functioning of the receptor, while a mutation of Asp^{321} in TM VII can only be accommodated if there is a Asp at position 90 [Blomenröhr *et al.*, 1997].

Extracellular loop (EL) I is the most conserved of the extracellular loops, followed by EL II and EL III. The carboxy-terminal end of EL II is highly conserved, while the amino-terminal end is completely unconserved (fig. 2.10). Glu^{301} of the mouse GnRH receptor (or the equivalent Asp^{302} of the human GnRH receptor) in EL III is important

for selectivity for Arg^8 of GnRH, seen in the mammalian GnRH receptors [Flanagan *et al.*, 1994]. As previous studies suggested that the amphibian pituitary was not selective for different GnRH agonists [Licht *et al.*,1984; 1987], it was surprising to note that the Glu in the corresponding position of the *X. laevis* receptor, was conserved (Glu^{288}, fig. 2.10). Pharmacological characterisation of the *X. laevis* GnRH receptor reveals that it does apparently have some discrimination for the Arg in position 8 since [Gln^8]GnRH (cGnRH I) was at least 20-fold less potent than [Arg^8]GnRH (mGnRH). Nevertheless it is clear that the Glu^{288} of the *X. laevis* GnRH receptor is not as powerful a determinant of ligand selectivity as in the mammalian receptor, because unlike in the mammalian receptors, cGnRH II and sGnRH, which lack the Arg in position 8, are far more potent than mGnRH (table 2.2). Interestingly, a proline residue (Pro^{303}) in the human GnRH receptor follows the crucial acidic residue, whilst a proline precedes the acidic Glu^{288} in the amphibian receptor. As proline residues are known to disrupt the secondary structure of proteins [Chou and Fasman, 1978] particularly α-helices, this change may drastically alter the conformation of the loop, and thus change the orientation of the acidic residue side chain and consequently the ligand selectivity. The crucial determinants of high ligand potency in the *X. laevis* receptor, in distinct contrast to mammalian receptors, is a Trp in position 7, as all analogues with this residue had high potency (table 2.2). Presumably there is a cognate interacting residue (or residues) in the *X. laevis* receptor.

Intracellular loop (IL) I of the *X. laevis* receptor is much shorter than that of the human receptor (5 amino acids in the frog, versus 12 amino acids in the human). The mammalian GnRH receptors have a longer IL I when compared to all other GPCRs characterised to date [Probst *et al.*, 1992]. This long IL I in mammals may have evolved to compensate for the lack of carboxy-terminal tail, which is absent in the mammalian receptors. The short IL I of the *X. laevis* receptor is in keeping with other GPCRs with intracellular carboxy-terminal tails. IL II is poorly conserved. The DRS motif at the border of TM III and IL II of the mammalian GnRH receptors, is a DRH in the amphibian receptor. The Asp and Arg residues, which are conserved in the amphibian receptor, have been shown to be important for receptor conformation, expression, agonist-induced activation and internalisation [Arora *et al.*, 1997]. The Arg residue (Arg^{139}) is thought to interact with the Asp in position 138 and the Ile at

position 143, which occurs one turn down in the α-helix of TM III, extending into IL III [Ballesteros *et al.*, 1998]. This interaction may position the Arg appropriately for the active and inactive receptor conformations [Ballesteros *et al.*, 1998]. The carboxy-terminus of IL III is highly conserved. This region is thought to be important for G-protein coupling and signal transduction. Ala^{261} of the human receptor, which aligns to Ala^{247} of the *X. laevis* receptor, is conserved. This residue has been shown to be crucial for G-protein coupling and receptor internalisation [Myburgh *et al.*, 1998].

The *X. laevis* GnRH receptor, like the catfish [Tensen *et al.*, 1997], goldfish (see chapter 4) and chicken [Y-M Sun *et al.*, in preparation] GnRH receptors has an intracellular carboxy-terminal tail. The carboxy-terminal tail of GPCRs has been implicated in receptor phosphorylation leading to desensitisation and possibly internalisation [for review, see Benovic *et al.*, 1988]. The mouse GnRH receptor shows no short-term homologous desensitisation [Davidson *et al.*, 1994(b)]. This phenomenon may have evolved to facilitate the LH-surge required for ovulation. The frog pituitary GnRH receptor, in spite of having a intracellular carboxy-terminal tail responds to continuous high dose administration of GnRH and does not exhibit desensitisation [for review, see Licht, 1990]. The carboxy-terminal tail has several serine and threonine residues, which are putative protein kinase C and protein kinase A phosphorylation consensus sites required for homologous desensitisation. The presence of the carboxy-terminal tail and lack of desensitisation in the amphibian is, therefore, enigmatic.

Several GnRH receptor agonists were tested for their ability to stimulate inositol phosphate production in COS-1 cells transiently transfected with the *X. laevis* GnRH receptor. mGnRH, sGnRH and cGnRH I have previously been shown to be equipotent in stimulating LH release from the amphibian pituitary [Licht *et al.*, 1987]. These results are not reflected in the inositol phosphate assays on the *X. laevis* GnRH receptor, in which sGnRH is approximately 20-fold more potent than mGnRH, while cGnRH I shows very low activity (fig. 2.7 A; table 2.2). The discrepancy between the bioassay results in the amphibian pituitary and the GnRH stimulated inositol phosphate response in the transfected COS-1 cells is unknown, but may be as a result of the sensitivity of the different assays used. cGnRH II showed an approximately 2000-fold higher ability to stimulate inositol phosphate production than

mGnRH (fig. 2.7; table 2.2). cGnRH II has previously been shown to bind amphibian pituitary homogenates with a higher affinity than buserelin, a mGnRH agonist [Fasano *et al.*, 1990] (fig. 2.7 C, table 2.2). [Trp^7, Tyr^8]GnRH, which is 2-fold more potent than cGnRH II, in stimulating LH release in the chicken pituitary [Millar *et al.*, 1989], had a lower potency than cGnRH II for the amphibian receptor (table 2.2). [His^5]GnRH also had a lower potency in stimulating inositol phosphate production, thus, indicating that the combination of the His^5, Trp^7, and Tyr^8, in cGnRH II is required for its high activity in the amphibian receptor. D-amino acid substitutions in position 6 of GnRH are thought to enhance the activity of naturally occurring GnRHs by constraining them in the active conformation (for review, see Sealfon *et al.*, 1997). These substitutions have no effect on the potency of cGnRH II but constraining mGnRH enhances its potency by almost 100-fold, as seen for Buserelin (fig. 2.7 B, C; table 2.2).

Antagonist 26 ([Ac-*D*-p-ClPhe^{1,2}, *D*-Trp^3, *D*-Lys^6, *D*-Ala^{10}NH$_2$]GnRH) behaved like a true antagonist, and completely inhibited both mammalian- and chicken GnRH II stimulated inositol phosphate production, but were less potent than in the mammalian receptor [ ] (fig. 2.8 C, D). The mammalian GnRH receptor antagonist 135-18 ([Ac-*D*-Nal^1, *D*-4-ClPhe^2, *D*-Pal^3, Ile^5, *D*-IsopropylLys^{6,8}, *D*-Ala^{10}NH$_2$]GnRH), however, was a partial agonist for the amphibian GnRH receptor, as it could not completely inhibit the mGnRH and cGnRH II stimulated inositol phosphate production, and weakly stimulated inositol phosphate production at high concentrations (fig. 2.8 A, B). This suggests that the amphibian GnRH receptor binding site for 135-18 must be different from that of the mammalian receptor and that interactions occur, which can stabilise the *X. laevis* GnRH receptor in the active conformation.

Despite the use of several different labelled GnRH analogues (^{125}I-[D-Ala^6, N-ME-Leu^7, Pro^9-NHEt]GnRH; ^{125}I-[His^5, D-Arg^6, Trp^7, Tyr^8]GnRH; and ^{125}I-[Ac-*D*-p-ClPhe^{1,2}, *D*-Trp^3, *D*-Lys^6, *D*-Ala^{10}NH$_2$]GnRH), no specific binding to the amphibian receptor could be shown in transfected COS-1 cells. Specific displacement of ^{125}I-labelled cGnRH II by cGnRH II and buserelin, has previously been shown in the pituitary of the green frog, *Rana esculenta* [Fasano *et al.*, 1990]. The lack of labelled ligand binding in the COS-1 cells expressing the *X. laevis* receptor, is either a result of poor expression of the receptor or due to a low affinity of the receptor for the

labelled ligands tested. Less than 1% specific binding of ^{125}I-labelled mammalian- or cGnRH II agonists (^{125}I-[D-Ala^6, N-ME-Leu^7, Pro^9-NHEt]GnRH; ^{125}I-[His^5, D-Arg^6, Trp^7, Tyr^8]GnRH) could be detected in *X. laevis* pituitary membranes (for results, see chapter 3). The lack of detectable receptor binding may, therefore, be as a result of the low expression of this receptor in the transfected cells or due to a low affinity binding.

The sequence variations seen in the receptor cDNA clones may be as a result of either the duplicate genes, which result from the tetraploid genome of this species or the result of mutations which were incorporated by the polymerase during amplification of the cDNA. The sequence was therefore confirmed by sequencing of the genomic clone. The gene structure of this receptor is highly conserved with that of the mammalian GnRH receptor genes [Zhou and Sealfon 1994; Fan *et al.*, 1994], and consists of three exons, divided by two introns. The amphibian has a third intron at –3, which is approximately 1.3 kbp. The size of the exon encoding the 5' untranslated region of this receptor is unknown. The adaptor primer ligated at position -216 of the start codon. Although this may indicate the transcription start site it cannot be confirmed without proper characterisation of the promoter region 5' to this, and may be an artifact of cDNA synthesis.

The cloning and characterisation of the amphibian pituitary GnRH receptor, has thus, provided some useful insights into the structure/function activity of GnRH receptors. This vertebrate receptor is substantially different from the mammalian receptors with regard to both structure and pharmacology. The amino acid sequence is 53% different from the human GnRH receptor with major changes in the amino-terminus, and both intracellular and extracellular loops, while the transmembrane domains have a higher conservation. The amphibian receptor has a different ligand selectivity for the naturally occurring GnRHs, possibly due to a change in the presentation of extracellular loop III. The mammalian GnRH receptor antagonist 135-18 is a partial agonist for this receptor, indicating that the low amino acid conservation possibly dictates changes in ligand recognition and receptor activation. The presence of an intracellular carboxy-terminal tail may indicate a different mechanism of receptor regulation, internalisation and desensitisation, which may be important for the amphibian pituitary response. In depth analysis of several of these changes has

already been initiated for the *X. laevis* GnRH receptor as well as the chicken GnRH receptor which has recently been cloned (Y-M Sun *et al.*, in preparation; Ott *et al.*, 1998)). The use of chimeric receptors as well as site-directed mutagenesis, where several changes are incorporated at once, may help to delineate co-ordinated amino acid changes and how they relate to receptor specificity, activation and regulation.

Chicken GnRH II-like peptides and a receptor selective for chicken GnRH II in amphibian sympathetic ganglia

3.1 Summary

GnRH has been demonstrated to be a neurotransmitter in amphibian sympathetic ganglia, where its release from the presynaptic nerve terminals reversibly inhibits M-current, a time- and voltage-dependent potassium current [Jan and Jan, 1980]. The occurrence of GnRH and GnRH receptors in sympathetic ganglia extracts was investigated. A peptide was detected in extracts of bullfrog (*Rana catesbeiana*) and African clawed frog (*Xenopus laevis*) sympathetic ganglia, which had identical immunoreactivity and retention times on high performance liquid chromatography as cGnRH II. Under the chromatographic conditions used, a second unknown peptide with cGnRH II immunoreactivity co-eluted with synthetic mammalian GnRH, but showed no cross-reactivity with the specific mGnRH antisera. The second unknown peptide was not hydroxy-proline cGnRH II or oxidised cGnRH II. To test the possibility of the presence of a receptor specific for cGnRH II in sympathetic ganglion neurons, competition binding of membranes extracted from the sympathetic ganglia of the two amphibian species was investigated with ^{125}I-labelled GnRH agonists. The binding of ^{125}I-[His^5,D-Arg^6,Trp^7,Tyr^8]GnRH (a cGnRH II agonist) to membranes from the sympathetic ganglia of both amphibian species was specific and had a higher affinity than cGnRH II, mGnRH and a mGnRH agonist, [D-Ala^6,NMe-Leu^7,Pro^9-NHEt]GnRH. These findings suggest that endogenous cGnRH II may play a role in synaptic transmission in the sympathetic ganglia via a receptor specific for cGnRH II. In an attempt to clone a GnRH receptor from these neurons RNA was extracted from sympathetic ganglia and several different techniques were utilised. A novel second GnRH receptor subtype was detected after PCR on genomic DNA, but a full-length cDNA for this isoform could not be found in the sympathetic ganglia cDNA. Despite the specific binding seen in membranes from sympathetic ganglia it is, therefore, not clear whether a second GnRH receptor subtype is expressed in these ganglia.

3.2 Introduction

The functional significance of the presence of more than one form of GnRH in any one species is unknown, but suggests a widespread function (see chapter 1). The best characterised role of GnRH is the regulation of the synthesis and release of gonadotropins from the anterior pituitary [for reviews, see Schally *et al.*, 1971; Kiesel, 1993]. GnRH does, however, have several other neuroendocrine roles, including a paracrine role in the gonads [for review, see Hsueh and Schaeffer, 1985], an autocrine role in certain tumours [Harris *et al.*, 1991], and synaptic transmission [Jan and Jan, 1980]. As described in chapter 2 the distribution and abundance of the two forms of GnRH found in amphibia has been well characterised [Rivier *et al.*, 1981; King and Millar, 1986; Sherwood *et al.*, 1986; Di Meglio *et al.*, 1991; Fasano *et al.*, 1993; Muske *et al.*, 1994; Licht *et al.*, 1994; King *et al.*, 1994(a); Collin *et al.*, 1995; Di Matteo *et al.*, 1996; Pinelli *et al.*, 1997]. These studies suggest that in amphibians, mGnRH, which is found predominantly in the hypothalamus, is the prime regulator of gonadotropin release, while cGnRH II, which predominates in the midbrain and hindbrain may have an additional role, possibly in neurotransmission.

GnRH has been demonstrated to be a co-neurotransmitter at the synapses in bullfrog sympathetic ganglia. Its release from the preganglionic nerve terminals generates a late, slow, excitatory postsynaptic potential [Jan and Jan, 1980]. The depolarisation is due to the inhibition of M-current, which is a time-and voltage-dependent potassium current [Adams and Brown, 1980; Brown and Adams, 1980; Jan and Jan, 1982; Adams *et al.*,1983], named from its inhibition by muscarinic acetylcholine receptor agonists. M-current was initially described in bullfrog sympathetic ganglia but has since been shown in many central and peripheral neurons [Brown, 1988] and rat sympathetic ganglia [Owen *et al.*, 1990]. M-current has a potential clamping effect on the neurons of the sympathetic ganglia and may be part of a negative feedback mechanism that regulates neuron excitability [Adams *et al.*, 1982; Jones, 1985; Kirkwood and Lisman, 1992]. Inhibition of this current may, therefore, allow the neurons to be more responsive to sympathetic inputs. M-current inhibition has been shown to have long term effects on neuron excitability [Kirkwood and Lisman, 1992]. The depolarisation results from a blockade of the M-current, via the interaction of a pertussis toxin insensitive G-protein with the M-channel [Pfaffinger, 1988; Lopez and Adams, 1989; Lopez, 1992; Stansfeld *et al.*, 1993]. Several second messenger systems have been investigated and although

some effects on M-current were observed, none have been directly implicated [Bosma and Hille, 1989; for review, see Adams and Brown, 1980]. It has, therefore, been proposed that the G-protein may interact directly with the M-channel, like the inwardly rectifying potassium channels of the heart [Breitwieser *et al.*, 1985]. GnRH has been shown to be a neurotransmitter in bullfrog sympathetic ganglia, although the endogenous isoform is unknown, cGnRH II is a candidate for several reasons [for review, see Adams and Brown, 1980]. Synthetic cGnRH II is a 100-fold more potent than [Trp^7, Leu^8]GnRH (sGnRH), and a 1000-fold more potent than mGnRH, [Gln^8]GnRH (cGnRH I) and [Tyr^3,Leu^5,Glu^6,Trp^7,Lys^8]GnRH (lamprey GnRH I), in its ability to inhibit M-current [Jones, 1987]. The agonist-dependent inhibition also desensitises to cGnRH II, but not to salmon GnRH [Jones 1987, for review, see Adams and Brown, 1980]. In order to investigate this, HPLC and RIA were used to determine whether cGnRH II is present in extracts of sympathetic ganglia. The possibility of the presence of a receptor specific for cGnRH II was also tested, using various analogues of GnRH in receptor binding assays on membranes from these neurons. The presence of a second GnRH receptor subtype in sympathetic ganglia was also investigated using PCR-based technologies.

3.3 Materials and Methods

3.3.1 Tissue Dissection

Tissue from the bullfrogs was supplied by Dr Y. Peng (University of Chicago, USA), and was prepared as follows: Adult bullfrogs were decapitated and the viscera removed. For the HPLC study, ganglia 7 through 10 along with spinal nerves 7 and 8 from 12 bullfrogs were removed (fig. 3.1). The tissue was bathed in 0 Ca^{2+}, 1 mM EGTA Ringer solution. The 0 Ca^{2+} Ringer was used to minimise the release of GnRH during the dissection. Connective tissue was removed from the ganglia and the spinal nerves in order to obtain the most highly concentrated nervous tissue possible. Tissues were pooled and lyophilised for HPLC. For the receptor binding assay, ganglia 7 through 10 from 12 bullfrogs were removed while the tissue was bathed in 0 Ca^{2+}, 1 mM EGTA Ringer solution. Again, connective tissue was removed from the ganglia. The ganglia were then placed in phosphate-buffered saline (PBS) on ice. Whole bullfrog brains without the pituitary, were removed from the telencephalon to the tectum. The brains were placed immediately in PBS, while the connective tissue enveloping the frog brain

was removed. The samples were then frozen on dry ice, lyophilised, and extracted as described below. Tissue from *X. laevis* was prepared as follows: adult *X. laevis* were pithed and the viscera removed, exposing the vertebral column. Ganglia 7 through 10 were excised and placed directly in PBS on ice. The cranial bone was then cut away to expose the brain (fig. 3.2) and the pituitary was removed and placed in PBS on ice. For the HPLC, the ganglia from 10 *X. laevis* were collected.

3.3.2 Tissue Extraction

Lyophilised tissue was extracted with 2N acetic acid containing 10^{-3} M phenylmethanesulfonylfluoride at 4°C using a Thomas homogeniser. The homogenates were centrifuged at 18,000 g for 1 h at 4°C, and the supernatants were lyophilised. Tissue extracts were reconstituted in water and sonicated prior to HPLC.

3.3.3 High-Performance Liquid Chromatography (HPLC)

HPLC analysis was performed using a Beckman model 421 equipped with a model 112 solvent delivery module and a model 153 UV detector. Samples were injected via a 2 ml loop onto a Vydac C-18 reverse-phase column (0.46 x 25 cm; 5 μm particle size). The mobile phase was 0.1% heptafluorobutyric acid (solvent A) and 60% acetonitrile in 0.1% heptafluorobutyric acid (solvent B). Elution conditions were: 32% B for 10 minutes, a linear gradient from 32% to 60% B over 30 minutes, followed by a linear gradient from 60 to 99% B over 5 minutes, and finally 99% B for 5 minutes. The flow rate was 1.5 ml/min. One-minute fractions were collected. Aliquots of tissue extracts and 20 ng of each of eight known GnRHs were chromatographed as standards. Following runs of synthetic peptides, the entire HPLC system was cleaned with several gradients of solvents A and B, as described above, and finally with acetonitrile, to ensure against contamination. Furthermore, prior to runs of biological material, blank column runs were monitored by radioimmunoassay to ensure that the HPLC system was not contaminated and that the peaks observed in biological runs were authentic. Column fractions were lyophilised, reconstituted in phosphate-buffered saline, and assayed in duplicate for immunoreactive GnRH using antisera 678, 1076, and 675.

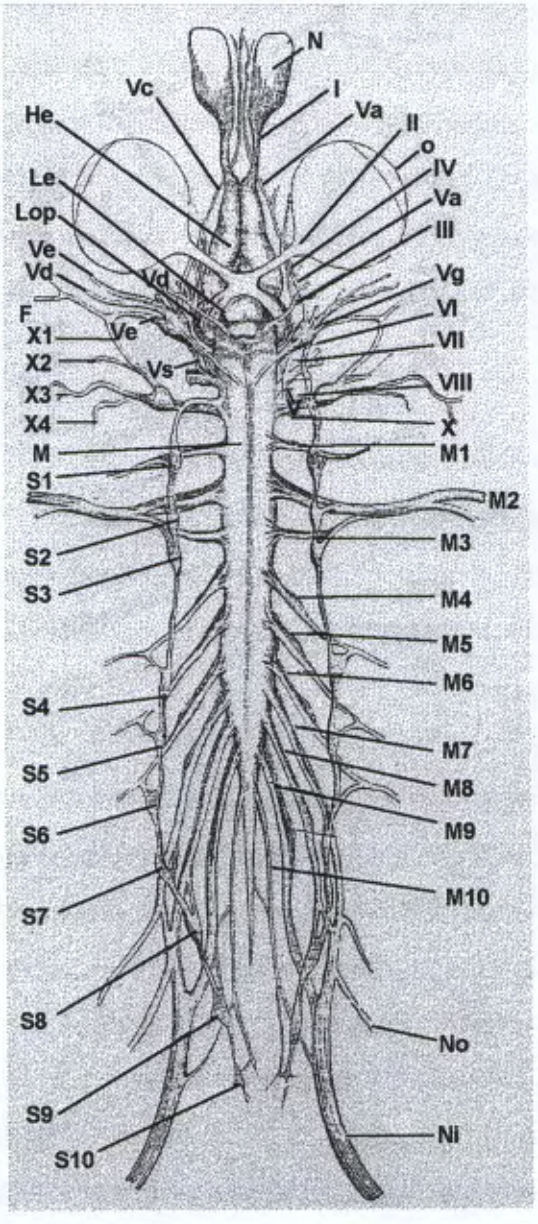


Figure 3.1. *Diagram of the nervous system of Rana esculenta from the ventral surface [Marshall, 1932]*

The sympathetic nervous system consists of a longitudinal nervous band on each side of the body, connected by branches with spinal nerves. Ganglia 7-10 (**S7-S10**) were removed from bullfrog and *X. laevis* for binding and HPLC studies.

F, facial nerve; **He**, cerebral hemisphere; **Le**, optic tract; **Lop**, optic lobe; **M**, boundary between medulla oblongata and spinal cord; **M1-10**, spinal nerves; **N**, nasal sac; **Ni**, sciatic nerve; **No**, crural nerve; **o**, eyeball; **S**, trunk of sympathetic; **S1-10**, the sympathetic ganglia; **Sp**, continuation of sympathetic into head; **I**, olfactory nerve; **II**, optic nerve; **III**, motor oculi; **IV**, fourth nerve; **V**, trigeminal and facial nerves; **Va**, ophthalmic branch of trigeminal; **Vc**, maxillary branch of trigeminal; **Vd**, mandibular branch of trigeminal; **Ve**, hyomandibular branch of facial; **Vg**, Gasserian ganglion; **Vs**, upper end of sympathetic trunk; **VI**, abducens nerve; **VII**, facial nerve; **VIII**, auditory nerve; **X**, glossopharyngeal and pneumogastric nerves; **X1**, ramus anterior of glossopharyngeal; **X2**, ramus posterior of glossopharyngeal; **X3-4**, branches of pneumogastic.

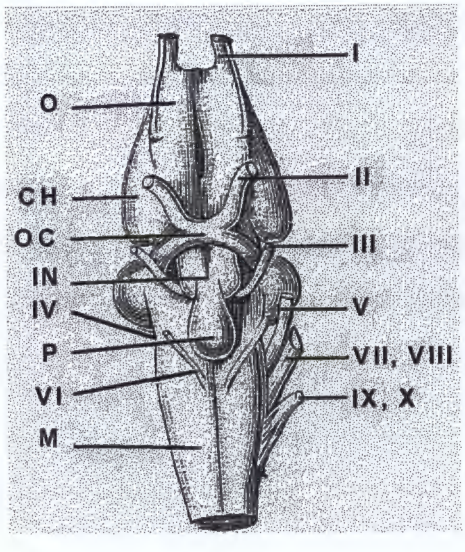


Figure 3.2. *Diagram of a frog brain from the ventral surface [Marshall, 1932]*

The cranial bone was cut away to expose the brain and the pituitary, **P**, which was then removed.

CH, cerebral hemisphere; **IN**, tuber cinereum; **M**, medulla oblongata; **O**, olfactory lobe; **OC**, optic chiasma; **P**, pituitary body, **I**, olfactory nerve; **II**, optic nerve; **III**, motor oculi nerve; **IV**, fourth nerve; **V**, trigeminal nerve; **VI**, sixth nerve; **VII** and **VIII**, combined root of facial and auditory nerves; **IX** and **X**, combined root of glossopharyngeal and pneomogastric nerves.

3.3.4 Oxidation of Chicken GnRH II

1μg of cGnRH II at 0.2μg/μl was oxidised in the presence of 50% hydrogen peroxide (H_2O_2, *Sigma*) for 1 h at room temperature.

3.3.5 Radioimmunoassay of GnRHs

Radioimmunoassays for GnRH were as previously described for cGnRH II [King and Millar, 1986]. Antiserum characteristics, in terms of antigen used, titre, sensitivity, and cross-reactivity with known GnRHs, are given in Table 3.1. cGnRH II antiserum 678 (in a heterologous radioimmunoassay using mGnRH tracer and standards) requires the NH_2- and COOH-termini for binding and cross-reacts with all known GnRHs. mGnRH antiserum 1076 is specific for mGnRH, and binds the middle of the molecule. Specific cGnRH II antiserum 675 recognises residues five to eight of the molecule. The amount of peptide present in the sympathetic ganglia was determined from the amount of immunoreactive GnRH present in the pooled extracts, divided by the total number of frogs used in the study.

Table 3.1 *Antisera Characteristics*

Peptides	*Antiserum*	*Titer*	*ED_{50}*		*Percentage cross-reactivity*				
			pg	*M*	*CI*	*CII*	*S*	*LI*	*Mh*
mGnRH	1076	1:50 000	17	100	59	0.4	0.3	<0.1	11
cGnRH II	678	1:40 000	25	100	416	81	473	3.1	23
	675	1:5 000	18	<0.1	<0.1	100	<0.1	<0.1	-

The Radioimmunoassays were homologous except for the radioimmunoassay where chicken GnRH II antiserum 678 was used with mammalian GnRH tracer. **M,** mammalian GnRH; **CI,** chicken GnRH I; **CII**, chicken GnRH II; **S**, Salmon GnRH; **LI**, lamprey GnRH I; **Mh**, hydroxyproline mammalian GnRH.

3.3.6 *Radioiodination of GnRH analogues*

[His^5,D-Arg^6,Trp^7,Tyr^8]GnRH and [D-Ala^6,NMe-Leu^7,Pro^9-NHEt]GnRH were iodinated according to an adapted chloramine-T method [Millar *et al.*, 1995]. Iodinated peptide was purified on a Vydac C18 reverse phase column (0.46 x 25 cm; 5 μm particle size) using a linear gradient of 0.01M ammonium acetate (NHAc), pH 4.6 to 60% CH_3CN/40% 0.01 M NHAc pH4.6. A profile of the elution of ^{125}I[His^5,D-Arg^6,Trp^7,Tyr^8]GnRH can be seen in figure 3.3. The specific activity for both iodinated peptides was approximately 1000 μCi/μg as determined by the self-displacement method [Millar *et al.*, 1989].

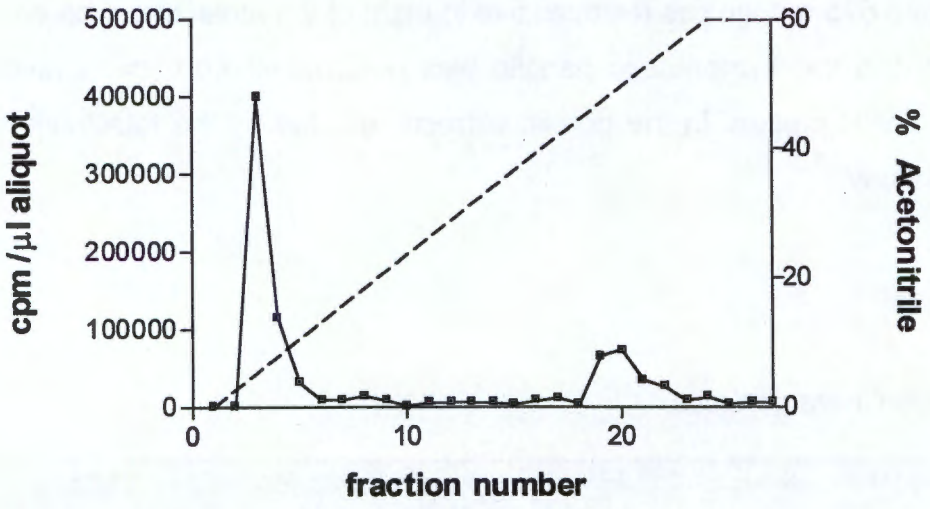


Figure 3.3. *Elution profile of ^{125}I[His^5, D-Arg^6, Trp^7, Tyr^8]GnRH.*
Radiolabelled C GnRH II agonist was eluted from a Vydac C-18 column with a linear gradient of acetonitrile in ammonium acetate (0.01 M, pH 4.6)

3.3.7 *Receptor Binding Assay*

^{125}I-[D-Ala^6,NMe-Leu^7,Pro^9-NHEt]GnRH (superactive mGnRH agonist) and ^{125}I-[His^5,D-Arg^6,Trp^7,Tyr^8]GnRH (superactive cGnRH II agonist), which are putative superactive analogues of two of the naturally-occurring GnRH peptides known to be present in amphibians, were used to study binding of GnRH to receptors in sympathetic ganglia of both amphibian species [Millar *et al.*, 1986(b); Millar *et al.*, 1989]. The freshly dissected sympathetic ganglia tissue was homogenised using a Dounce homogeniser (Wheaton) in ice-cold binding buffer (10 mM HEPES, 1 mM EDTA and 0.1% fatty acid free BSA). The homogenate was centrifuged for 30 minutes at 10,000 g at 4°C. The binding assay was performed as previously described [Millar *et al.*, 1986(b)]. The membrane pellet

was weighed and resuspended in binding buffer such that each experimental tube contained one third sympathetic ganglion or pituitary equivalent, for the *X. laevis* and for the bullfrog one sixth sympathetic ganglion equivalent. Data points were determined in triplicate. The mouse αT3 pituitary gonadotrope cell line, which expresses mammalian GnRH receptors [Windle *et al.*, 1990], was used as a positive control, and sciatic nerve was used as a putative negative control. Non specific binding was determined by displacement with either 10^{-5} M mGnRH agonist or cGnRH II (see figure legends). Non specific binding was subtracted from total binding to give specific binding. The total radioactivity of the labelled peptide added to each tube was approximately 80,000 cpm.

3.3.8 Cloning of a second GnRH receptor subtype

The degenerate primers JH5s and JH6a$_2$ were used to amplify the second subtype of receptor from genomic DNA (see Chapter 2, Materials and Methods). This clone was labelled and used as a probe to screen a *X. laevis* genomic DNA library, for detailed methods, see section 2.3.6.

3.3.9 Cloning of a partial cDNA

Total RNA was prepared from *X. laevis* hindbrain, pituitaries and sympathetic ganglia by extraction with guanidium thiocynate [Chomczynski and Sacchi, 1987], as described in section 2.3.2. Gene specific primers were designed to the PCR product amplified using JH5s and JH6a$_2$, p*Xl*b.1 (see appendix 7.7). These primers were used to amplify cDNA synthesised from 1 μg of DNase I digested sympathetic ganglia total RNA, using the Marathon cDNA synthesis kit (Clonetech). cDNA synthesis was monitored by the incorporation of [α-^{32}P]dCTP (1 μCi/μl, Amersham). cDNA was amplified using the Marathon system (Clonetech) and products were probed with the nested primer *Xl*1b.4s. These methods are described in detail in Chapter 2 (see section 2.3.7). Positive products were subcloned into the pMOS*Blue* T-vector (Amersham) and recombinant colonies analysed by colony PCR or colony hybridisation to a suitable probe (see section 2.3.5).

3.3.10 Cloning of cDNA from the gene

The exon trapping method developed by Buckler *et al.* (1991) was utilised in an attempt to isolate transcribed sequences from the genomic clone of *Xl*b. The genomic clone in EMBL 4 was digested with BamH1 endonuclease to yield three

products of approximately 8 kbp, 6 kbp and 3 kbp. The latter two endonuclease fragments were cloned into the dephosphorylated BamH1 site of the pSPL3 vector (see appendix 7.8a)(Exon Tapping System; Gibco BRL). Two clones of each type, designated pSPL3.2a and pSPL3.2b for the 6 kbp clones and pSPL3.3a and pSPL3.3b for the 3 kbp clones, were prepared and co-transfected in combinations into COS-1 cells. The combinations are described in table 3.2. Plasmid DNA for transfection was prepared using the Wizard DNA purification system (Promega). 24 h prior to transfection, cells were plated at a density of 3 x 10^6 cells on 10 cm poly-*D*-lysine coated dishes (Corning), in DMEM containing 10% foetal calf serum, penicillin (0.2 U/ml) and streptomycin (100 μg/ml). Cells were transfected with a total of 15 μg of plasmid DNA (7.5 μg of each clone) in serum-free DMEM for 4 h using an adapted DEAE-dextran method [Keown *et al.*, 1990], with a 50 minute chloroquine (200 μM) treatment and a 2 minute 10% dimethylsulphoxide shock [Luthman and Magnusson, 1983; Lopata *et al.*, 1984]. Cells were grown for 48 h after transfection in DMEM containing 10% foetal calf serum and penicillin/streptomycin, prior to extraction of total RNA. Cells were removed from plates using 0.04% EDTA in 10 ml of PBS, collected by centrifugation for 10 minutes at 1000 rpm and resuspended in 500 μl of denaturing solution (see section 2.3.2). Total RNA was extracted as described above and resuspended at a concentration of approximately 1 μg/μl in DEPC water. cDNA synthesis and two rounds of nested PCR were performed as described in the kit protocol. The products of the PCR reactions were analysed by agarose gel electrophoresis and Southern blotting with ^{32}P labelled p*Xl*b.1. Clone p*Xl*b.1 was labelled to a specific activity of approximately 7 x 10^8 cpm/μg using [α-^{32}P]dCTP (3000 Ci/mmol) and the Megaprime labelling kit (both Amersham). Any positive PCR reactions were then subjected to an additional round of PCR using gene specific primers designed to p*Xl*b.1 (see appendix 7.7). Products were analysed once again by agarose gel electrophoresis and Southern blotting. Any positive products were excised from a low melting point agarose gel (FMC BioProducts) and subcloned into the pMOS*Blue* T-vector (Amersham). Recombinant colonies were sequenced (T7 Sequenase version 2.0, Amersham).

Table 3.2. *Combinations of plasmids, co-transfected for exon trapping analysis*

Combination	Plasmids co-transfected
1	pSPL3.2a;pSPL3.3a
2	pSPL3.2b;pSPL3.3a
3	pSPL3.2a;pSPL3.3b
4	pSPL3.2b;pSPL3.3b
5	Untransfected COS-1 cells

3.3.11 Sympathetic ganglia cDNA library construction and screening

Sympathetic ganglia poly A^+ RNA was purified from 100 μg of total RNA by adsorption to oligo(dT) cellulose (Sigma) according to a modified method first described by Aviv and Leder (1972). A Stratagene (La Jolla, California) ZAP-cDNA synthesis kit was used to make the cDNA libraries from 1 μg of mRNA. The sympathetic ganglion library was amplified to a titre of 1.4 x 10^5 pfu/μl of phage lysate. 10^6 plaques of the amplified library were screened as described in the ZAP-cDNA kit protocol, with ^{32}P-labelled mouse GnRH receptor cDNA. The cDNA was labelled to a specific activity of approximately 7 x 10^8 cpm/μg using [α^{32}P]dCTP (3000 Ci/mmol) and the Megaprime labelling kit (both Amersham). Labelled probes were purified from unincorporated deoxynucleotides using a Sephadex G-50 spin column, equilibrated with 100 μl of STE (10 mM tris-HCl, pH 8; 1 mM EDTA; 100 mM NaCl). The hybridisation conditions were as described in section 2.3.4. The library was also screened with labelled β-actin cDNA to determine the quality of the library. β-Actin was labelled using the Megaprime labelling kit (Amersham).

3.4 Results

3.4.1 Chicken GnRH II is a GnRH isoform present in sympathetic ganglia

Detection of the amount of GnRH by radioimmunoassay (NH_2-and COOH-terminus-directed antiserum 678) in the sympathetic ganglia was as follows: bullfrog (266 pg per animal) and *X. laevis* (43 pg per animal) (Dr J. King). In HPLC of the bullfrog sympathetic ganglion extract, two peaks were revealed by the NH_2- and COOH-terminus-directed antiserum 678, and these co-eluted with synthetic mGnRH and cGnRH II (fig. 3.4.A). Specific mGnRH antiserum 1076 showed no cross-reactivity, thus mGnRH is not present in the sympathetic ganglion (fig. 3.4.C). cGnRH II antiserum 675 cross-reacted with both peaks, and quantification of each of the peaks

was approximately equal to that quantified with antiserum 678 (fig. 3.4.B). Peak II is likely to be cGnRH II, while the identity of peak I is unknown. The possibility of this peak being one of two less hydrophobic chemically modified forms of cGnRH II; namely oxidised cGnRH II and hydroxyproline cGnRH II, was tested. Under standard HPLC conditions; hydroxyproline cGnRH II (Zymogenetics) co-eluted with synthetic mGnRH, while oxidised cGnRH II, co-eluted with synthetic cGnRH II. In heterologous RIAs, however, antisera 678 and 675, showed similar cross-reactivity (38% and 31%) to oxidised cGnRH II (fig.3.5 A and B). Under the same conditions antiserum 675 showed a higher cross-reactivity with hydroxyproline cGnRH II (37.5%) than antiserum 678 (2%). Neither hydroxyproline cGnRH II or oxidised cGnRH II, thus, exhibited characteristics of the peptide in peak I. The *X. laevis* sympathetic ganglion extract showed similar findings (data not shown).

3.4.2 GnRH receptors in the sympathetic ganglia showed a specificity for a chicken GnRH II agonist

In order to determine the receptor specificity in the sympathetic ganglia, binding of ^{125}I-mGnRH agonist and ^{125}I cGnRH II agonist (see materials and methods for peptide definitions), to membranes from bullfrog sympathetic ganglia and *X. laevis* sympathetic ganglia and pituitary were tested in comparison to αT3 cells (figs. 3.6 and 3.7, respectively). The specific binding of ^{125}I-cGnRH II agonist was lower than the specific binding of ^{125}I-mGnRH agonist, for the αT3 cells, in both experiments, with a ratio of 0.52 and 0.3 (figs. 3.6 and 3.7, respectively). In contrast, the specific binding of ^{125}I-cGnRH II agonist was higher than the specific binding of ^{125}I-mGnRH agonist for the bullfrog sympathetic ganglia (fig. 3.6), with a ratio of 2.67. The differential binding of the *X. laevis* sympathetic ganglia to labelled cGnRH II and mGnRH agonists is consistent with the results obtained with the bullfrog sympathetic ganglia. The binding ratio of the two agonists was 3.9 respectively. The *X. laevis* pituitary membranes show no selectivity for the two different labelled agonists. The concentrations of various GnRH agonists to inhibit 50 percent of the binding of ^{125}I-cGnRH II agonist (the IC_{50}) in bullfrog and *X. laevis*, are compared in table 3.3. The cGnRH II agonist had an IC_{50} of approximately 10^{-8} M in the sympathetic ganglia of both amphibian species (fig. 3.8, table 3.3). The affinity of cGnRH II was slightly lower with IC_{50}s for the two species between 10^{-7} M and 10^{-6} M. The binding of the mGnRH agonist and mGnRH was much lower, with the relative affinities differing in the two species.

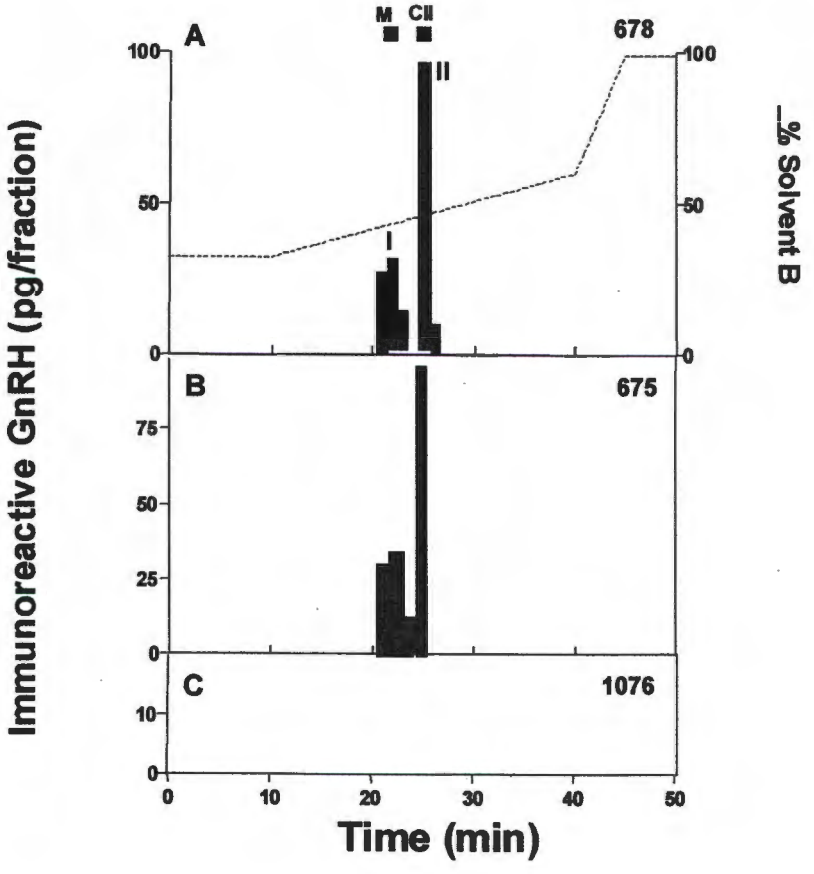


Figure 3.4. Reverse-phase HPLC of an aliquot of bullfrog sympathetic ganglion extract and radioimmunoassay with (**A**) NH_2-and COOH-terminus-directed cGnRH II antiserum 678, (**B**) cGnRH II antiserum 675, and (**C**) mGnRH antiserum 1076. Immunoreactive GnRH was eluted from a Vydac C-18 column with a stepwise gradient of acetonitrile in 0.1% heptafluorobutyric acid. Elution positions of synthetic mGnRH (M) and cGnRH II (CII) are indicated. Retention times of other known GnRHs under these conditions are: chicken I (11 min), catfish (11 min), mammalian hydroxyproline (16 min), lamprey I (23 min), dogfish (29 min), and salmon (30 min) (This was done by Dr J. King).

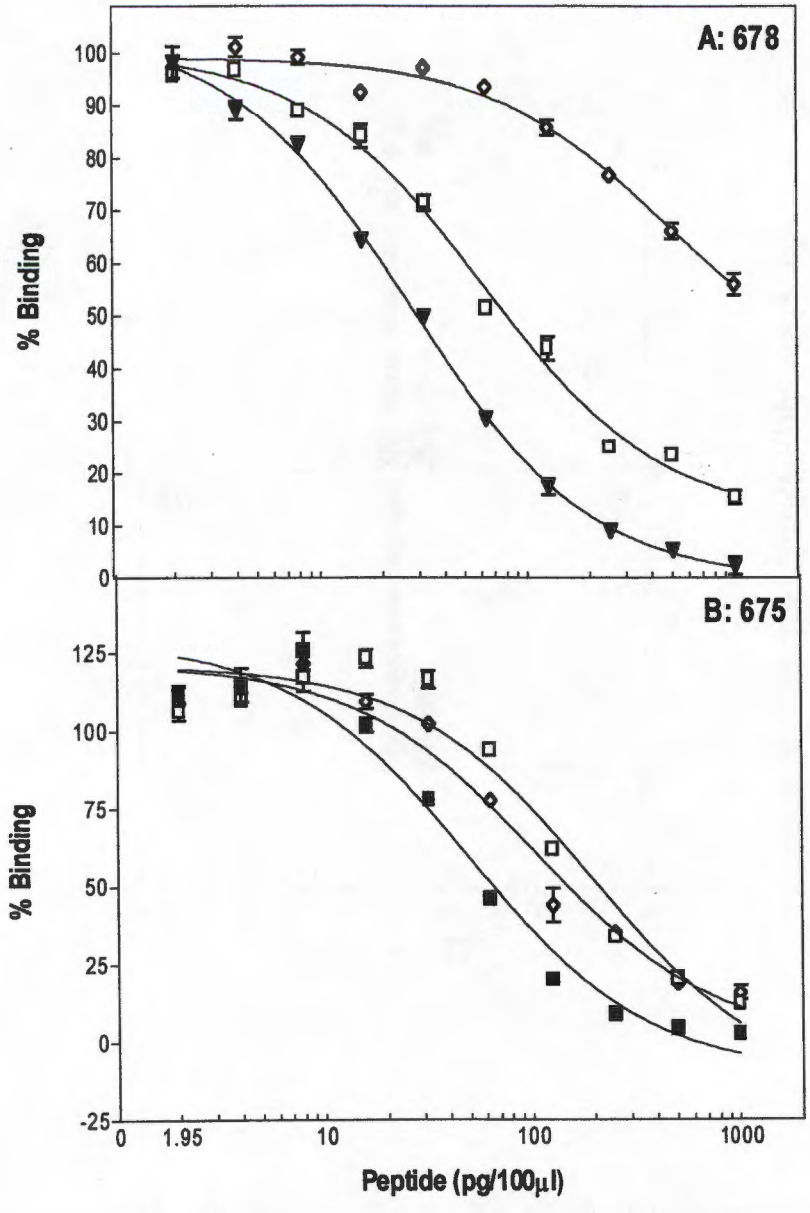


Figure 3.5. Heterologous radioimmunoassays of (A) antiserum 678 with ^{125}I labelled mammalian GnRH and (B) antiserum 675 with ^{125}I labelled chicken GnRH II. Percent binding was calculated as a percent of the maximum possible binding. Mammalian GnRH (▼), Chicken GnRH II (■), oxidised chicken GnRH II (□) and hydroxyproline chicken GnRH II (◆). Values are mean ± SE.

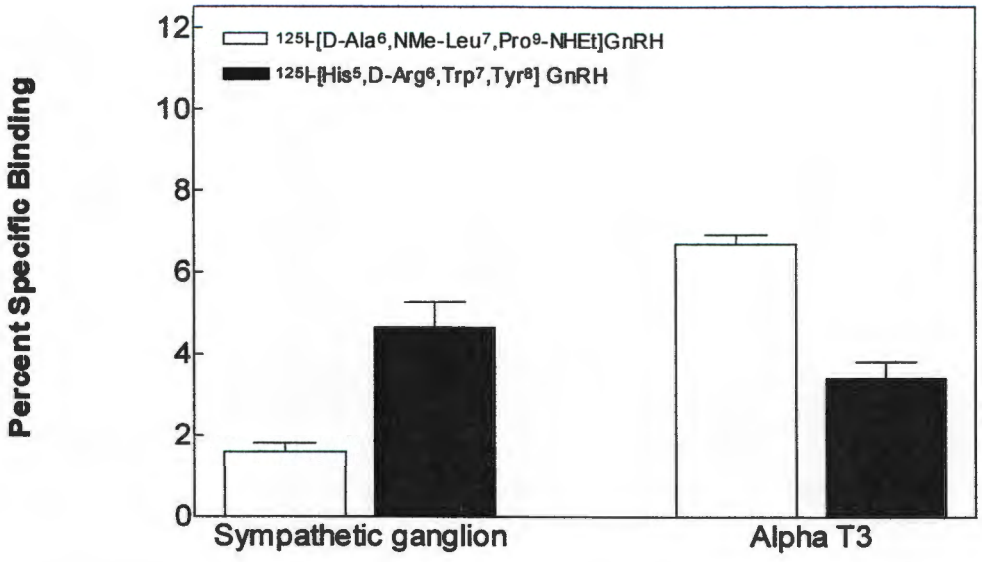


Figure 3.6. Binding of (□) ^{125}I-mammalian GnRH agonist and (■) ^{125}I-chicken GnRH II agonist to membranes from bullfrog sympathetic ganglion and αT3 cells. Triplicate aliquots containing 1/6 tissue equivalents were incubated with labelled peptide for 1 h at 4°C. Data are expressed as percentage binding, which was calculated as percentage specific binding (cpm)/tissue (mg)/total cpm added. Nonspecific binding was the binding (cpm) at 10^{-5} M [D-Ala^6,NMe-Leu^7,Pro^9-NHEt]GnRH (□) and [His^5,D-Arg^6,Trp^7,Tyr^8]GnRH (■). Values are mean ± S.D.

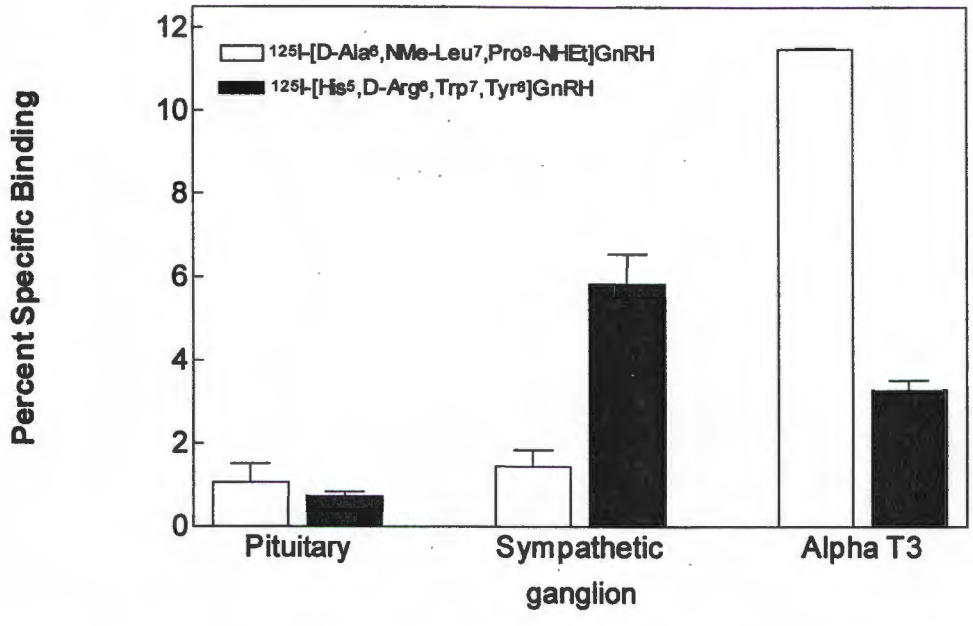


Figure 3.7. Binding of (□) ^{125}I-mammalian GnRH agonist and (■) ^{125}I-chicken GnRH II agonist to membranes extracted from *X. laevis* pituitary, sympathetic ganglion and sciatic nerve, and αT3 cells. Triplicate aliquots containing 1/3 tissue equivalents were incubated with labelled peptide for 1 h at 4°C. Data are expressed as percentage binding which was calculated as specific binding (cpm)/tissue (mg)/total cpm. Nonspecific binding was the binding (cpm) at 10^{-5} M [D-Ala^6,NMe-Leu^7,Pro^9-NHEt]GnRH (□) and [His^5,D-Arg^6,Trp^7,Tyr^8]GnRH (■). Values are mean ± S.D.

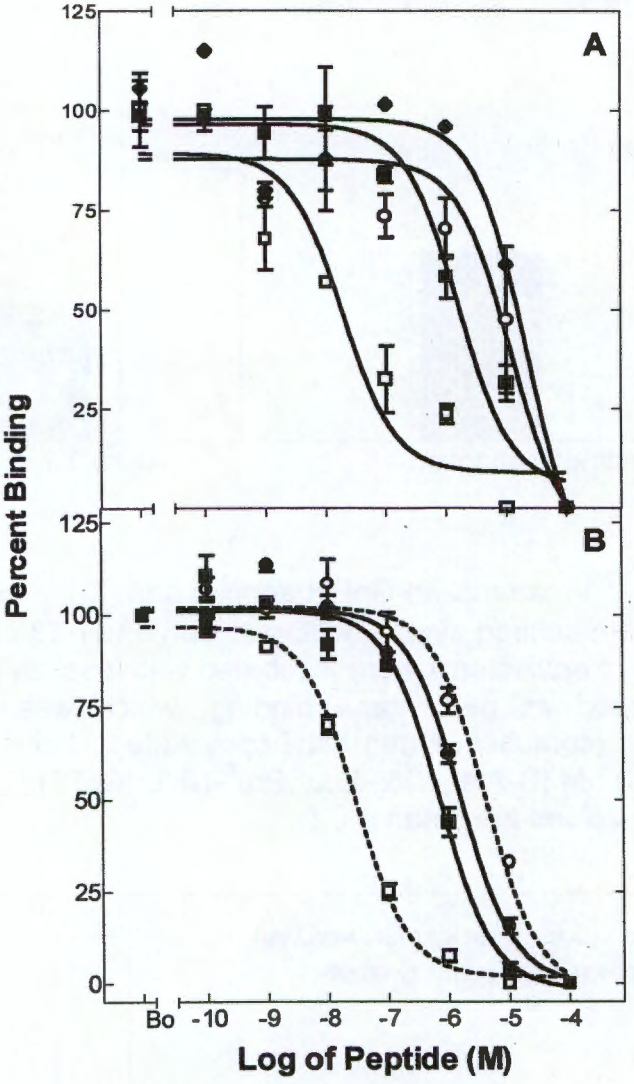


Figure 3.8. Competition binding curves of the chicken GnRH II agonist (^{125}I-[His^5,D-Arg^6,Trp^7,Tyr^8]GnRH), on membranes from sympathetic ganglia of (**A**) bullfrog and (**B**) *X. laevis* , in the presence of increasing concentrations of unlabelled [His^5,D-Arg^6,Trp^7,Tyr^8]GnRH (□), cGnRH II (■), mGnRH (●), [D-Ala^6,NMe-Leu^7,Pro^9-NHEt]GnRH (○). Percentage binding was calculated as percentage binding (cpm)/ specific binding (cpm), where binding is determined as nonspecific binding subtracted from the binding. Nonspecific binding (NSB) was the binding (cpm) at 10^{-5} M [His^5,D-Arg^6,Trp^7,Tyr^8]GnRH. Maximum binding (Bo) was determined in the absence of unlabelled peptide. Values are mean ± S.D. Data reduction was performed using GRAPHPAD PRISM^{TM}.

Table 3.3. *Inhibtion constants*

Peptide	IC_{50} of binding in bullfrog sympathetic ganglia membranes	IC_{50} of binding in X. laevis sympathetic ganglia membranes
[His^5,D-Arg^6,Trp^7,Tyr^8]GnRH	$1.8x10^{-8}$ M	$2.8x10^{-8}$ M
[His^5, Trp^7,Tyr^8]GnRH (chicken GnRH II)	$1.6x10^{-6}$ M	$7.4x10^{-7}$ M
[D-Ala^6,NMe-Leu^7, Pro^9-NHEt]GnRH	$1.2x10^{-5}$ M	$2.1x10^{-6}$ M
mammalian GnRH	$2.3x10^{-5}$ M	$1.4x10^{-6}$ M

Inhibition constants for the competitive binding of [His^5,D-Arg^6,Trp^7,Tyr^8]GnRH, chicken GnRH II, mammalian GnRH, [D-Ala^6,NMe-Leu^7,Pro^9-NHEt]GnRH, and hydroxyproline chicken GnRH II with ^{125}I-[His^5,D-Arg^6,Trp^7,Tyr^8]GnRH to membranes from sympathetic ganglia of *X. laevis* and bullfrog. Data reduction was performed using GRAPHPAD PRISM™.

3.4.3 Amplification of genomic DNA yielded a second GnRH receptor isoform

PCR using the degenerate primers JH5s and JH6a$_2$, resulted in the amplification of two putative receptor subtypes, which showed homology to the cloned mammalian GnRH receptors (see section 2.4.1). These clones, p*Xl*a.1 and p*Xl*b.1 both showed a 35% identity at the amino acid level, to the human GnRH receptor (see appendices 7.3 and 7.7). Clone p*Xl*b.1, however, had a two amino acid insertion in the region of extracellular loop 3 (fig. 3.9). The full-length cDNA for p*Xl*a.1 was cloned from the pituitary and is described in chapter 2. Clone *Xl*b.1 was used to screen a *X. laevis* genomic DNA library. Five positive plaques were isolated after secondary and tertiary screening. Restriction enzyme and Southern blot analysis showed that the genomic clones are approximately 16 kbp.

```
                1                                                  39
                JH5s                                               JH6a2
Human     giwywf DPEMLN..RL SDPVNHFFFL FAFLNPCF dpliy
Xla       giwywf QPEMIN..QT PEYLNHSLFL FGLLHTCT dpliy
Xlb       giwywf PPEMLTEEKV PPVLSHILFL FGLLNTCL dpliy
```

Figure 3.9. Alignment of the PCR products, *Xl*a and *Xl*b obtained with the degenerate primers, JH5s and JH6a$_2$ to the amino acid sequence of the corresponding region of the human GnRH receptor. *Xl*a and *Xl*b have a 35% amino acid identity to the human GnRH receptor, extracellular loop III. *Xl*b has a two amino acid insertion. Primer sequences are shown in italics, conserved residues are shown in red and conservative changes are in blue.

3.4.4 Amplification of sympathetic ganglion cDNA

Sympathetic ganglion cDNA was synthesised as described in materials and methods and the quality was assessed by agarose gel electrophoresis. (fig. 3.10). No products could be seen in the same size range as the control cDNA. Low molecular weight smears could, however, be seen. Despite the low molecular weight of the first strand cDNA products, they were, nevertheless, used in nested PCR with primers designed to p*Xl*b.1 (see appendix 7.7). After a second, nested PCR, the products were probed with an internally nested primer (fig. 3.11). Products from PCR amplifications with annealing temperatures of 64°C and 68°C hybridised to the primer. The products amplified with an annealing temperature of 64°C were slightly larger, with a faint product at approximately 900 bp, than those amplified at 68°C, where the largest product was approximately 600 bp (fig. 3.11). The 900 bp product was subcloned and 136 recombinant colonies screened by colony PCR followed by Southern blotting or colony hybridisation, both using the labelled nested gene specific primer (*Xl*1b.4s, see appendix 7.7). DNA was prepared from 22 recombinant colonies, which hybridised to the primer. Only 11 of these clones contained inserts in the expected size range. Figure 3.12 shows a summary of the sequencing results. Analysis of the clones, MSG.1 - MSG.6 showed homology to the *X. laevis* pituitary GnRH receptor and the human GnRH receptor (fig. 3.12). One of the clones MSG.7, which had a different sequence after the valine (fig. 3.12, see arrow) may have a retained intron, as no open reading frame could be found. This valine, therefore, appears to be the exon/intron boundary. This boundary would align identically to the boundary of intron II in the human GnRH receptor gene [Fan *et al.*, 1994] and the intron II boundary of the *X. laevis* pituitary GnRH receptor (see chapter 2). The sequence with the longest open reading frame (MSG. 4) with homology to the human GnRH receptors as well as the *X. laevis* pituitary GnRH receptor, possibly ends at the border of transmembrane domain V and extracellular loop III, which does not align to the exon/intron boundaries of any of the characterised GnRH receptor genes [Zhou and Sealfon, 1994; Fan *et al.*, 1994; and chapter 2]. This truncation, probably represents an incomplete cDNA transcript. As none of the cDNA clones contained a full-length receptor, cDNA synthesis was repeated to increase the specificity, using a gene specific primer designed to p*Xl*b.1 (*Xl*b.1a, see appendix 7.7) instead of the oligo dT cDNA synthesis primer supplied by the Marathon kit. The double stranded cDNA was run on an agarose gel following second strand synthesis, but no cDNA

could be seen after a one week exposure of the autoradiograph. This may be due to the low incorporation of [^{32}P]dCTP as a result of only the one species of RNA being synthesised into cDNA. PCR amplification was, therefore, continued. Following two rounds of nested PCR amplification, and Southern blotting, no products containing the nested primer *Xl*b.4s sequence could be seen.

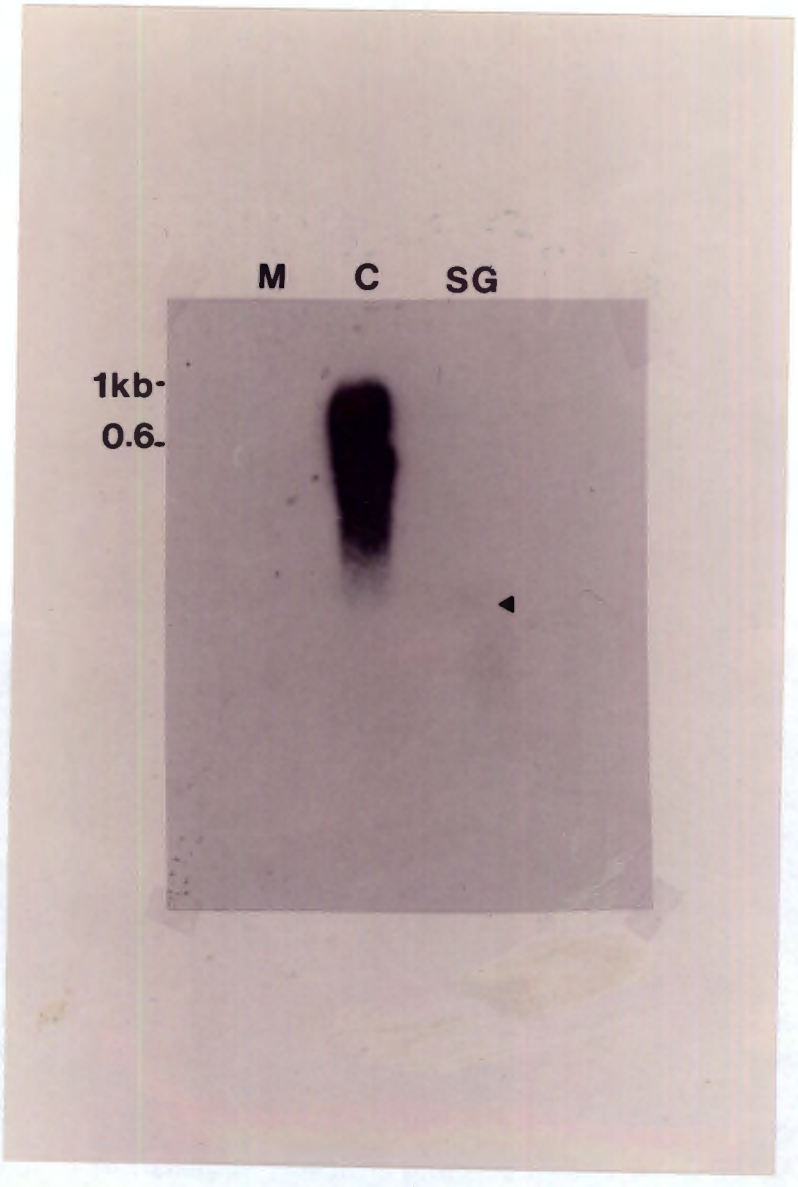


Figure 3.10. Autoradiograph showing the *X. laevis* sympathetic ganglia cDNA after the first strand synthesis (SG). A positive control was provided with the Marathon cDNA amplification kit (Clonetech) (C). M represents the MW marker. Only very low molecular weight products could be seen (see arrow).

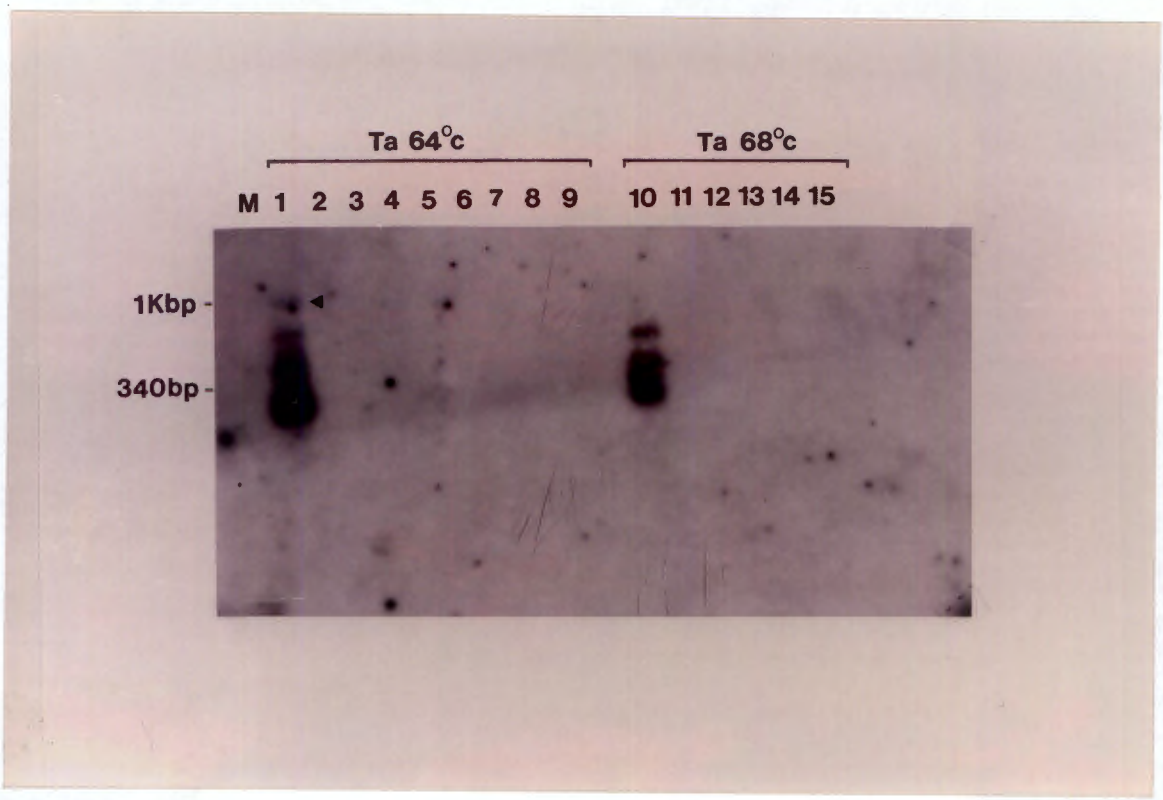


Figure 3.11. Autoradiograph of the Southern blot after two rounds of nested Marathon PCR on the *X. laevis* sympathetic ganglia cDNA, using the gene specific primers designed to Xlb (see appendix 7.7, primers *Xl*1b.1a and *Xl*1b.2a for 5' amplification and *Xl*1b.4s and *Xl*1b.3s for 3' amplification). The blot was probed with [γ^{32}P]dATP end-labelled *Xl*1b.4s. Lanes 1 - 3 and 10 – 12 represent 5' amplification, lanes 5 - 7 represent 3' amplification , lanes 4, 7 - 9, 13 - 15 are negative controls, where no DNA was added. **Ta:** annealing temperature; **M:** MW marker; **1:** sympathetic ganglia cDNA amplified with adaptor- and antisense gene specific primers (ie. 5' RACE); **2:** sympathetic ganglia cDNA amplified with adaptor primers only; **3:** sympathetic ganglia cDNA amplified with gene specific primers only; **4:** no cDNA control; **5:** sympathetic ganglia cDNA amplified with adaptor- and sense gene specific primers (ie. 3' RACE); **6:** sympathetic ganglia cDNA amplified with gene specific primers only; **7 - 9:** no cDNA controls with different primer combinations; **10 - 13:** sympathetic ganglia cDNA as for lanes 1 –3; **13 – 15:** no cDNA controls, with different primer combinations. A 900 bp product was seen after PCR with an annealing temperature of 64°C (see arrow), while after amplification at 68°C, the largest product was approximately 600 bp.

3.4.5 3' Amplification of sympathetic ganglia cDNA reveals that the Xlb receptor has an intracellular carboxy-terminal tail.

Three sense gene specific primers were designed to p*Xl*b.1, for amplification of sequences 3' to p*Xl*b.1. The membrane used in figure 3.11 was stripped by the addition of boiling 0.5 % SDS and probed with the primer *Xl*1b.1a, which is an internally nested primer for the 3' amplification reaction (see appendix 7.7). Two high molecular weight products of 3 kbp and > 10 kbp hybridised to the primer (fig. 3.13). The 3 kbp product was subcloned, and 30 recombinant colonies screened by colony PCR, followed by Southern blotting. Six positive clones were obtained. The smallest of these, pSG3'1, of approximately 500 bp was sequenced. The sequence of this clone revealed the partial sequence of the intracellular carboxy-terminal tail (see appendix 7.9).

3.4.6 PCR amplification of pituitary, hindbrain and sympathetic ganglion cDNA using primers to pXlb.1.

The expression of the alternative subtype of the GnRH receptor, (p*Xl*b.1) may occur in tissues other than the sympathetic ganglia and it is possible that both the subtypes of receptor are expressed in the pituitary. Alternatively, the expression of this receptor, may co-localise with the expression of cGnRH II in the amphibian brain [King *et al.*, 1994(a)]. Pituitary cDNA was, therefore, subjected to amplification (fig. 2.4) using the primers designed to the p*Xl*b.1 GnRH receptor subtype, as well as cDNA synthesised from total RNA extracted from the hindbrains of several adult male *X. laevis*. Sympathetic ganglion cDNA was also used as a reference. Following three rounds of nested PCR amplification at an annealing temperature of 64°C, the PCR products were subjected to Southern blotting with the labelled internally nested primer, *Xl*1b.3a. The results can be seen in figure 3.14. Only the pituitary gave clear low molecular weight products of approximately 200 - 500 bp, which hybridised to the labelled primer. Slight smears can be seen in the products amplified from sympathetic ganglion and hindbrain cDNA. The PCR on the pituitary cDNA was repeated, but with variable annealing temperatures, ranging from 62°C to 68°C, and variable concentrations of cDNA, in order to optimise the conditions so as to obtain higher molecular weight products, possibly containing the full length cDNA. Although higher molecular weight products were obtained, no discreet bands could be seen on the blot. The products appeared to be smears ranging from approximately 2 kbp to

16 kbp. The lower part of the smear was excised from an agarose gel the DNA extracted from the agarose and subcloned. 66 recombinant colonies were screened by colony hybridisation, of which 20% were positive. DNA was extracted from positive colonies, and colonies with inserts in the correct size range were sequenced with *Xl*1b.2a primer. Five of the clones, gave completely unrelated sequences, while a sixth clone gave the correct sequence, but with the same intron sequence which was obtained in the sympathetic ganglion clones (MSG.7) (fig. 3.12).

3.4.7 Exon trapping

Products obtained after the initial PCR on the cDNA, extracted from COS-1 cells transfected with the various combinations of pSPL3 subclones (table 3.2) were subjected to agarose gel electrophoresis followed by Southern blotting (fig. 3.15). Products were digested with and without BstX1 restriction enzyme in order to eliminate products resulting from vector splicing (see appendix 7.8b). All the products gave slight smears. These products were then amplified in two nested PCR reactions using the gene specific primers, *Xl*1b.1a and *Xl*1b.2a (see appendix 7.7), with the vector specific primer $dUSD_6$ (see appendix 7.8a and b). The products were then blotted onto nitrocellulose and probed with the third nested primer *Xl*1b.4s (fig. 3.16). Only cDNA from combination number 3 (table 3.2) hybridised to the labelled primer. The products were approximately 200 bp -1 kbp in size, but no discreet bands could be seen after the Southern blot. These products were subcloned. DNA was prepared from 34 recombinant clones and analysed by Eco R1 and Pst 1 restriction enzyme digestions, followed by Southern blotting. Several of the clones gave identical digestions, so only seven of the clones were sequenced. Although the exon III and exon II appear to be spliced together, like the marathon sympathetic ganglia clones (fig. 3.12), analysis of regions more 5' to this gave unidentified sequence with no open reading frames and no sequence homology to the mammalian receptor. The one clone, which did have an open reading frame gave a sequence with a 96.4% nucleotide sequence identity in a 140 bp region, with the *X. laevis* genes for 18 S, 5.8 S and 28 S ribosomal RNAs (see appendix 7.10).

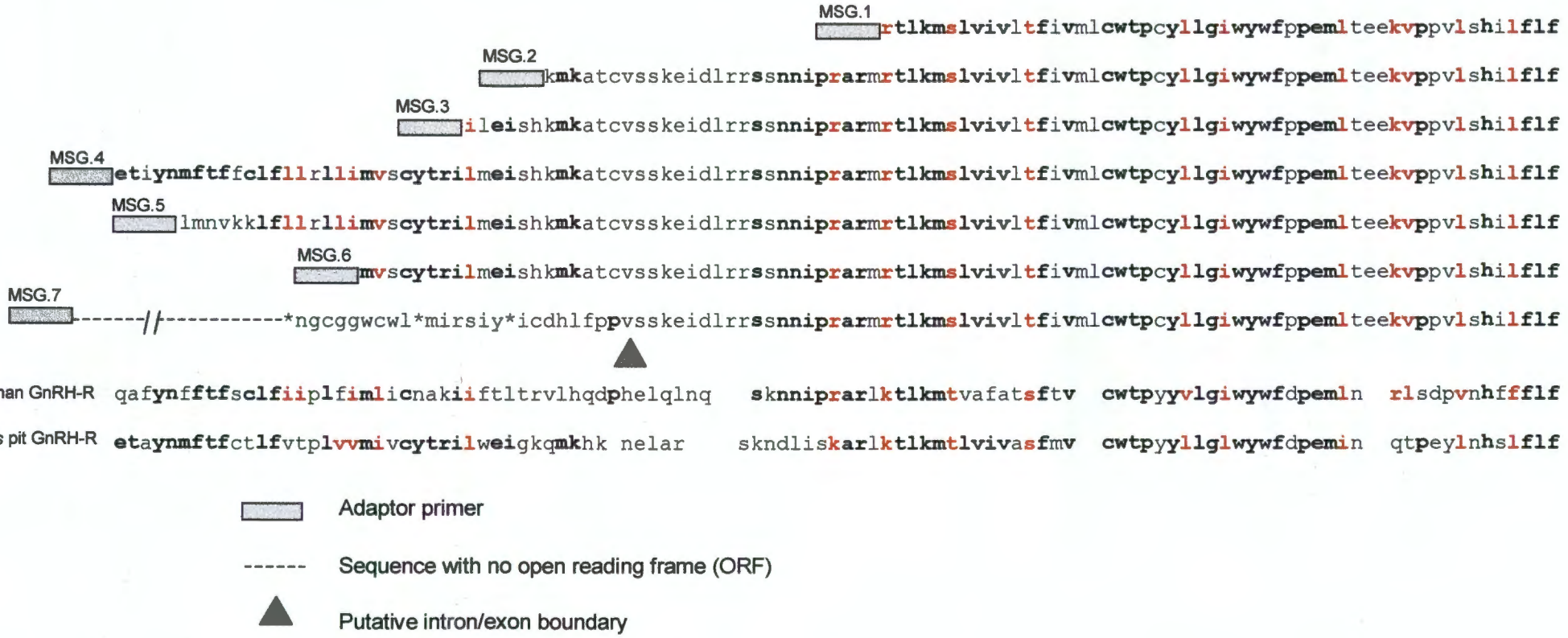


Figure 3.12. Summary of the sequences obtained from the subclones of the Marathon (Clonetech) PCR reaction on sympathetic ganglia cDNA. Clones are numbered from 1 to 7, but several clones of each were sequenced. There is a putative inton/exon boundary at the valine residue, shown, with a triangle. Residues shown in bold are conserved with the human GnRH receptor and/or the *X. laevis* pituitary GnRH receptor. Residues shown in red are conservative changes. Dashed lines represent sequences of varying lengths containing no open reading frames.

3.4.8 cDNA library screening yielded no positive clones

The sympathetic ganglion cDNA library was screened, using mouse GnRH receptor cDNA. No positive plaques were obtained after screening 10^6 plaques. The quality of the library was tested by probing with β-actin cDNA. One positive plaque was obtained per 1000 pfu. The cDNA library was also screened with the genomic fragments, p*Xl*a.1 and p*Xl*b.1, but no positive plaques were obtained.

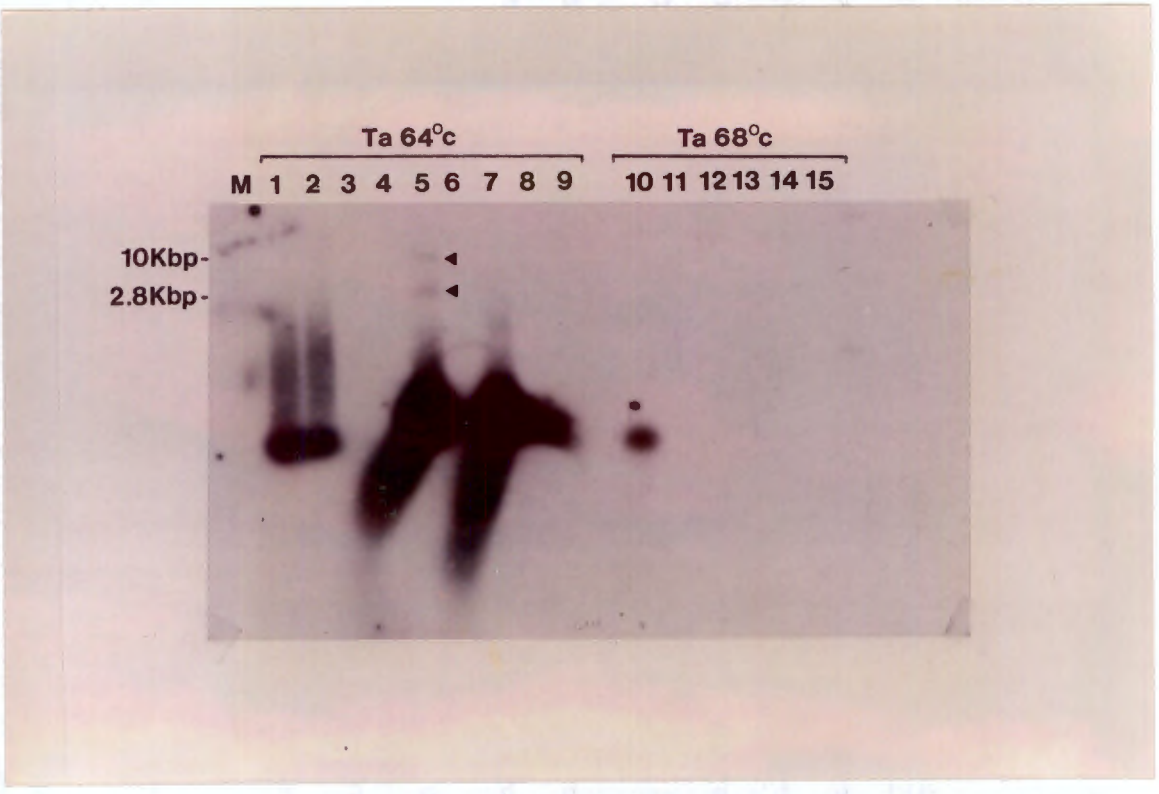


Figure 3.13. See figure 3.11 for gel loading. The membrane from figure 3.11 was stripped and probed with [γ^{32}P]dATP end-labelled *Xl*1b.1a, in order to see if any 3' amplification products were obtained. Two clear products of approximately 2.8 and 10 Kbp are seen in lane 5, (see arrows) which is sympathetic ganglia cDNA amplified with adaptor- and sense gene specific primers. High background bands are seen in lanes 1, 2 and 6 – 10 due to the overlap of primers used for PCR amplification and probing.

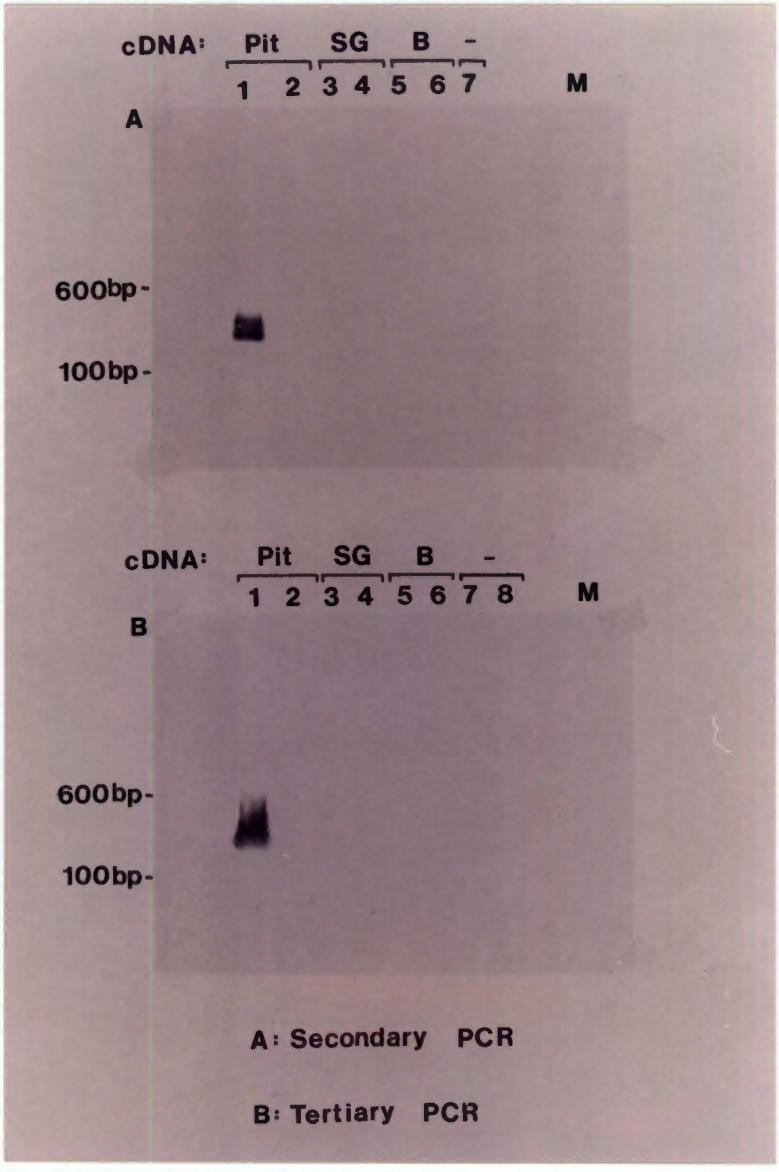


Figure 3.14. Autoradiograph of the Southern blot showing the *X. laevis* pituitary (Pit, 1, 1/10 dilution of cDNA; 2, 1/100 dilution of cDNA), sympathetic ganglia (SG 3, !/10 dilution of cDNA; 4, 1/100 dilution of cDNA) and hindbrain (B, 5. 1/10 dilution of cDNA; 6, 1/100 dilution of cDNA) after secondary and tertiary PCR amplifications with primers to pXlb.1. (appendix 7.7). The blot was probed with [γ^{32}P]dATP end-labelled Xl1b.4s. M, represents the MW marker while lanes 7 and are the water blanks.

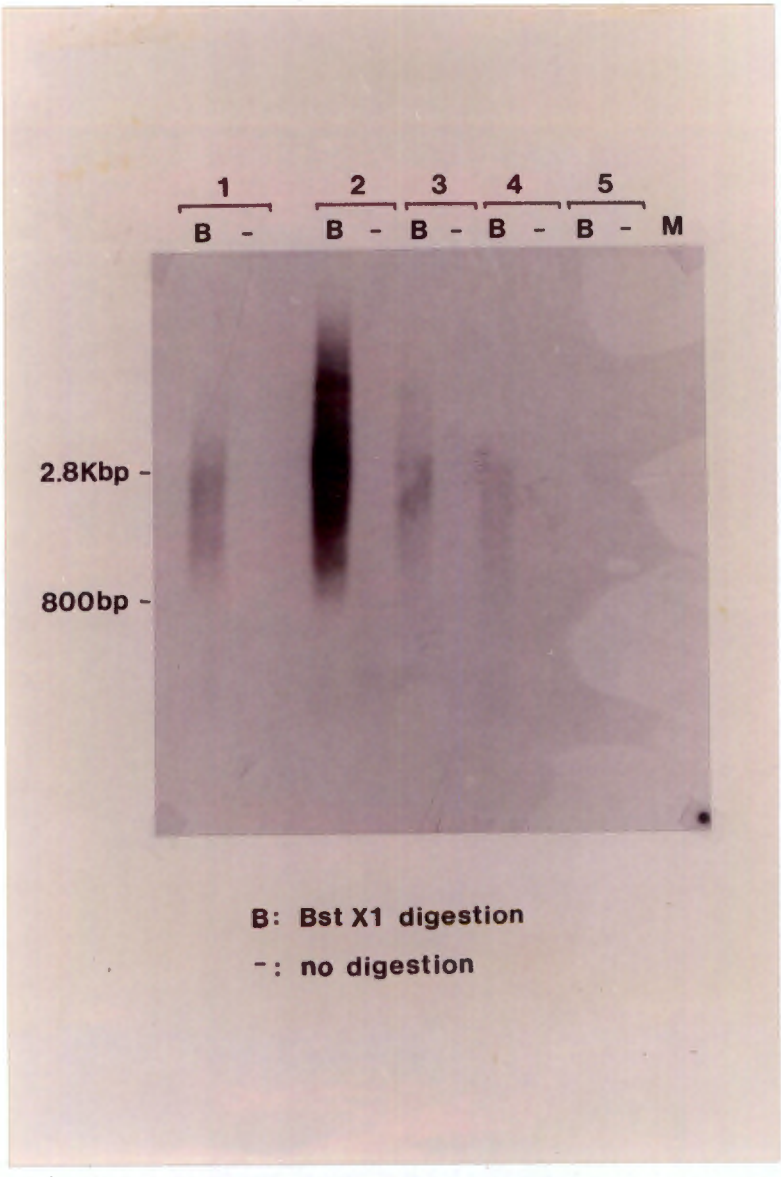


Figure 3.15. Autoradiograph of the Southern blot after amplification of exon trapped cDNA with the vector specific primers, SD_6 and SA_2 (appendix 7.8a and b), showing the 5 combinations of transfection (table 3.2) with and without BstX1 digestion. Membrane was probed with [γ^{32}P]dATP end-labelled *Xl*1b.4s. M, represents the MW marker

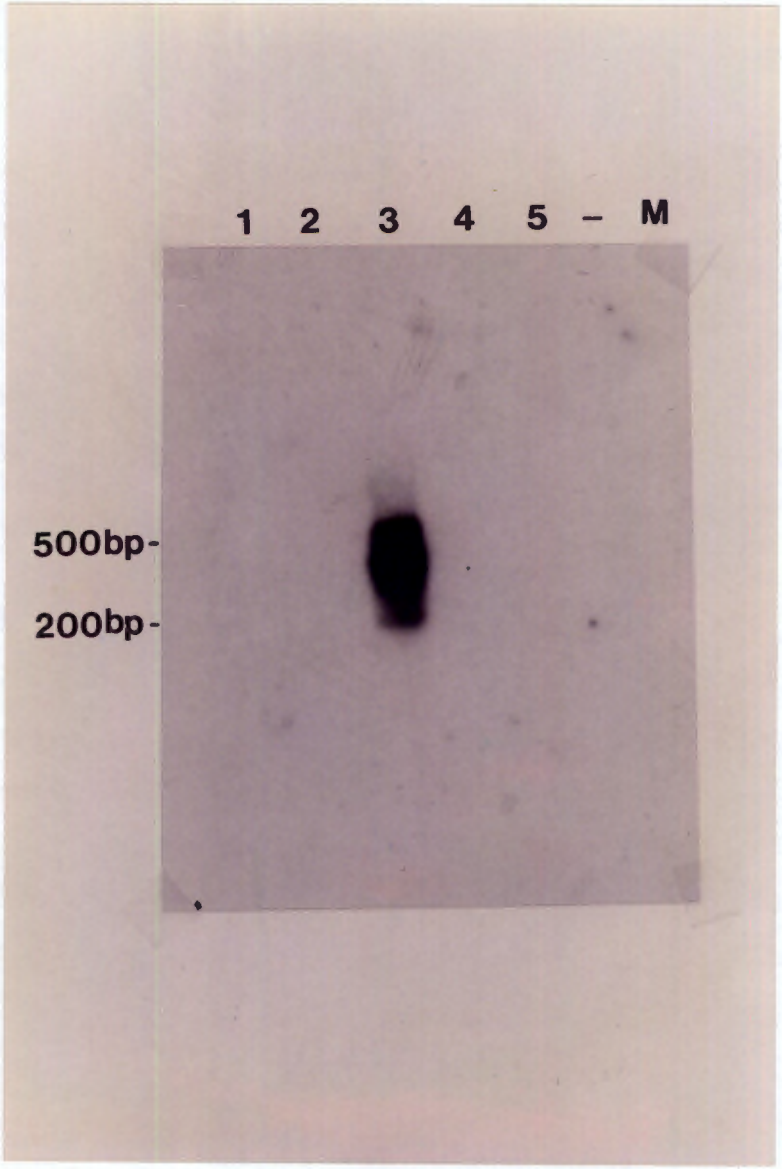


Figure 3.16. Autoradiograph of the Southern blot after secondary amplification of BstX1 digested, exon trapped cDNA with the gene specific primer to p*Xl*b.1, *Xl*1b.1a in combination with the vector primer $dUSD_2$ (appendix 7.7 and 7.8a and b), showing the 5 combinations of transfection (table 3.2). Membrane was probed with [γ^{32}P]dATP end-labelled *Xl*1b.4s. M, represents the MW marker.

3.5 Discussion

The electrical consequence of the release of GnRH in the bullfrog sympathetic ganglia is the inhibition of the M-current, which makes the ganglionic neuron highly excitable due to the increased membrane impedance. Unlike the short effect of acetylcholine release (lasting milliseconds) from the same set of nerve terminals, release of GnRH affects the membrane excitability for many minutes. Thus, during these minutes, the ganglia cells change from linear input-output relays to highly non-linearly amplifying synapses. The long-lasting effects of release of a neuropeptide as a transmitter in modulating a neuron's subsequent responses make this form of neuropeptide transmission an important candidate for a role in learning and memory in the nervous systems.

In this study we have detected the presence of immunoreactive cGnRH II in sympathetic ganglia of two amphibian species, *R. catesbeiana* and *X. laevis*, which has identical properties to synthetic cGnRH II. As GnRH has been shown to be a neurotransmitter in the bullfrog [Jan and Jan, 1980], it is very likely that the endogenous isoform of GnRH, used as a neurotransmitter in this system was cGnRH II. This is consistent with the electrophysiological studies where exogenous cGnRH II was found to be the most potent inhibitor of M-current [Jones, 1987]. The fact that less cGnRH II (43pg per animal) was detected in the ganglia of *X. laevis* than in *R. catesbeiana* can be accounted for by the difference in the sizes of the two species. Although electrophysiological data on M-current inhibition in *X. laevis*, is required, to conclusively prove the involvement of cGnRH II in this form of neurotransmission, it is likely that the effect on M-current by this peptide is similar to that in the bullfrog.

A second, unidentified, immunoreactive form of GnRH was also found in the sympathetic ganglia of both amphibian species. This peptide is not mGnRH as it showed no cross-reactivity with the specific mGnRH antiserum. It is also not a degradation product of cGnRH II, as it cross-reacted with the NH_2- and COOH-terminus directed antibody. It is possible that this peptide could be a chemically modified form of cGnRH II, because of their similar reactivity with the two cGnRH II antibodies or a novel form with sufficient common epitopes to result in considerable cross-reaction. Two possible forms of modified cGnRH II were tested, namely hydroxyproline cGnRH II and oxidised cGnRH II. Hydroxylation of cGnRH II, like the hydroxylation of mammalian

GnRH, may be a post-translational enzymatic modification for regulation of the cGnRH II response [Gautron *et al.*,1992]. The oxidation of cGnRH II may be an artefact of the extraction procedure. Both of these peptides would be less hydrophobic than cGnRH II and thus should elute earlier in the HPLC gradient. Neither of them proved to be candidates, due to the HPLC retention times and relative cross-reactivity with the different specific antisera. An unidentified form of GnRH has previously been detected in the *X. laevis*, but this form was very hydrophobic and, therefore, could not account for the earlier-eluting isoform [Licht *et al.*, 1994(a)]. The identity of the peptide in peak I is, therefore, unknown, and may be a novel form of GnRH, or a chemical modification of cGnRH II. Chemical modifications of GnRH arising during extraction, lyophilisation and chromatography in acetonitrile have previously been shown to occur as a result of artefacts of the HPLC procedure [King *et al.*, 1988].

The binding of the cGnRH II analogue to the sympathetic ganglia membranes of both amphibian species is specific as it can be displaced with unlabelled peptide. The inhibition coefficient (IC_{50}) of this binding was 10 nM, which is approximately 10-fold higher than that obtained for the suppression of M-current [Jones, 1987]. This difference is consistent with the amplification seen in signal transduction [Strickland and Loeb, 1981]. The differences in binding of the αT3 gonadotrope cell line with the two different ligands is anticipated as the mammalian GnRH pituitary receptors (fig. 3.6 and 3.7), are known to show a high fidelity for mGnRH when compared to other GnRH isoforms [Millar *et al.*, 1986(b), for review, see King and Millar, 1995]. The molecular structure of the sympathetic ganglion receptor to which cGnRH II binds is not known, but this study provides strong evidence for it to be significantly different from the cloned mammalian pituitary GnRH receptors [for review, see Stojikovic *et al.*, 1994]. The binding of both the mammalian and chicken II GnRH agonists in the pituitary of the *X. laevis* was low (fig. 3.7). A low binding of this peptide in the pituitary of the green frog has been previously reported [Fasano *et al.*, 1990], but no binding with the GnRH receptor from *X. laevis* pituitary could be detected in COS-1 cells (chapter 2). The amphibian pituitary GnRH receptor also shows a high selectivity for cGnRH II, compared to mGnRH analogues (chapter 2), but this receptor was not expressed in the sympathetic ganglia, as no specific products were obtained after reverse transcriptase PCR on sympathetic ganglia cDNA with primers to the *Xl*a receptor subtype.

We attempted to clone a receptor, which may correspond to the receptor which binds to cGnRH II in the sympathetic ganglia. Although a second subtype of receptor with homology to the *X. laevis* pituitary receptor as well as the cloned mammalian GnRH receptors was cloned from genomic DNA, no full length cDNA could be isolated from sympathetic ganglia cDNA using several PCR-based techniques. Two types of clones were, however, identified, with differences in the sequence after the putative exon/intron boundary. One had no open reading frame and may have been intron sequence, while the other had an open reading frame, with homology to the GnRH receptors, but was not a full-length receptor.

This putative receptor subtype does not represent a duplicate gene of the GnRH receptor isolated from *X. laevis* pituitary cDNA, which may result from the tetraploid genome *of X. laevis*, as the amino acid identity of this clone to the GnRH receptor isolated from pituitary cDNA is low (51%). There is, however, a possibility that clone *Xl*b may be a pseudogene, which arose from a duplication of the receptor thereby explaining the lack of a full length receptor and inconclusive splicing results from the exon trapping analysis of the genomic clone. This receptor may have been active in an ancient amphibian predecessor, but became redundant later in evolution. There are, however, several arguments against this possibility. All the sequences of the subclones except one which align to exon III and exon II of the *X. laevis* pituitary GnRH receptor, are open reading frames, with significant amino acid identity to both the human GnRH receptor and the *X. laevis* pituitary GnRH receptor. Only one clone contained sequence, which after the putative exon/intron boundary was not an open reading frame. At this region of sequence divergence, there is a typical intron donor site [Mount, 1982], therefore, adding evidence that this sequence is intronic. Further evidence for the existence of this second receptor comes from PCR analysis of extracellular loop III of lizard (*Agama atra*) genomic DNA by Dr E. Rumbak. Using identical conditions to those used to amplify the two genomic clones from *X. laevis* DNA, two clones were isolated from lizard DNA. One clone, Liz.1 had a high amino acid sequence identity to the *X. laevis* pituitary clone (76%), while the other, Liz.2 had a high amino acid identity to the *X. laevis Xl*b clone (78%). This clone also had the two amino acid insertion in the sequence of extracellular loop III when aligned to the mammalian, catfish and *X. laevis* pituitary GnRH receptors (fig. 3.9). The lizard clone, Liz.2, also had the two adjacent prolines, which were not present in the other pituitary GnRH receptors (fig. 3.9). A

human clone with homology to this lizard clone, Liz.2 and the *X. laevis* clone, *Xl*b was identified in the human genome data base (Millar *et al.*, submitted). The human clone had a high amino acid identity at 81% and 73% with the lizard and *X. laevis* clone *Xl*b respectively, and had the two adjacent proline residues, despite lacking the two amino acid insertion. These three receptors (*Xl*b, Liz.2 and the human clone), therefore, appear to be related. It is thus very unlikely that a receptor gene, which arose prior to the evolutionary divergence of reptiles, amphibians and mammals and which has been highly conserved in the region of extracellular loop III, is an inactive pseudogene.

The expression of *Xl*b receptor is, however, unknown. Sympathetic ganglion cDNA was extensively analysed using library screening as well as the sensitive Marathon (Clonetech) reverse transcriptase PCR. The sympathetic ganglion library gave reasonable expression with the ubiquitously expressed β-actin cDNA probe, despite the low titre, even after amplification (1.4×10^5 pfu/μl). The library was initially screened with the mouse GnRH receptor cDNA clone. This was, however, prior to the isolation of the two PCR clones generated from genomic DNA, *Xl*a and *Xl*b. Subsequent analysis of the homology of these clones to the mammalian GnRH receptor clones revealed a low homology (<45%), a probe with such a low homology would not hybridise under the conditions used to screen the library. The library was, therefore, screened with the genomic clones *Xl*a and *Xl*b. No positive clones were identified. The expression of these receptors may, thus, be too low to be represented in the one million plaques screened. The fact that no positive plaques were identified in the sympathetic ganglion library may, therefore, be as a result of the low initial titre of the library, or the low expression of this receptor in these neurons. The Marathon (Clonetech) PCR with primers designed to *Xl*b on sympathetic ganglia revealed a receptor, which did not contain the full length cDNA, but did appear to be processed in these neurons. Two splice variants were, however, obtained, one with an open reading frame with sequence homology to the cloned GnRH receptors, while the other had no open reading frame and no sequence homology, and may, therefore, be intron sequence. This unprocessed cDNA may have been due to a small amount of contaminating genomic DNA, or incompletely spliced RNA. The full length clone of *Xl*a was isolated from pituitary cDNA (Chapter 2), and was not expressed in the sympathetic ganglia, as determined by reverse transcriptase PCR. No full length, processed clones were isolated after Marathon PCR (Clonetech), this may have been due to the low average

molecular weight of the sympathetic ganglia cDNA (fig. 3.10.). cDNA synthesis was, therefore, repeated, but a gene specific primer was substituted for the oligo dT primer supplied with the synthesis kit. After nested PCR, the products were analysed. No specific products were obtained. Pituitary and hindbrain cDNA were also analysed for expression of this receptor. Again unprocessed cDNAs were identified, possibly indicating a lack of expression in these tissues. PCR products may have been as a result of a small amount of contaminating genomic DNA, but no direct conclusions on the expression of this second receptor subtype can be made. The genomic clone of *Xl*b was, therefore, used in an attempt to clone the full-length processed cDNA of this receptor. *X. laevis* genomic library, which was supplied by Dr T. Sargent (NIH, USA) was screened and five genomic clones isolated. The genomic clone was digested into smaller fragments, cloned into a splicing vector and transiently transfected into COS-1 cells. The cells were allowed to grow for three days, prior to the extraction of total RNA. cDNA was synthesised and amplified using PCR with primers designed to the splicing vector in combination with gene specific primers designed to *Xl*b. Although preliminary results seemed hopeful, a detailed analysis of several clones did not reveal any further sequence information. Because gene specific primers to the known regions of sequence were used in combination with a nested probe, clones corresponding to this region of the receptor were automatically enriched, while others, possibly corresponding to the unknown 5' sequences, which may have been present, would be un-identified. Due to the many variables in this Exon Trapping (Gibco BRL) method, such as orientation of the original clones and the possible lack of other 5' exons I decided to work with the regions of the receptor, with known sequences. It would, therefore, be easy to monitor the success of the method, by Southern blotting with a nested probe, for example, and if successful, it would be possible to return to the original cDNA for amplification and sequence analysis, but due to the presence of unprocessed cDNA no further sequence analysis was done. The one subclone generated by PCR amplification with a combination of gene specific primers to *Xl*b and the splicing vector showed a high nucleotide sequence homology (96.4%) for a significant length (140 bp) to *X. laevis* 28 S, 18 S and 5.8 S RNA (see appendix 7.10). This may be a result of a splicing artifact, generated by the splicing to an adjacent gene encoding the ribosomal RNAs. No direct conclusions could, therefore, be made from the exon trapping results.

Several approaches were, therefore, used in an attempt to clone the full-length cDNA of this receptor. The results seem to provide evidence that the full-length transcript for this receptor may not be expressed in the sympathetic ganglia, despite the occurrence of partially processed transcripts in these neurons. The possibility, therefore, exists, that this gene may be a pseudogene and is not expressed. As discussed above, there are several arguments against this, such as the occurrence of a highly homologous receptor in the lizard, *Agama atra*, and the human. A completely unprejudiced approach may, therefore, be required to clone this receptor. While it is tempting to assume that this receptor is the receptor responsible for the cGnRH II-induced inhibition of M-current in the sympathetic ganglia, seen in physiological studies, this assumption may be unfounded and the receptor may have a different function and may, therefore, be expressed in alternative, discreet brain areas. Although M-current has been described in rat [Owen *et al.*, 1990] it is not known whether it is a conserved mechanism for controlling neuron excitability in all vertebrates, and one can only speculate that this receptor is present in other vertebrate species. The fact that homologous receptor subtypes are also present in reptiles and mammals does not illuminate the situation, as this amphibian receptor has an unknown function and expression. A systematic approach using a diverse range of tissues may, therefore, be required. Studies using *in situ* hybridisation have been initiated with collaborators, but no results are available yet.

While no conclusions on the structure of this receptor can be made with the limited sequence information available, a few interesting observations can be addressed. From the sequence which is available, this cDNA appears to encode a G-protein coupled receptor with a 47%, 51% and 53% amino acid identity to the human, catfish and *X. laevis* pituitary GnRH receptors, respectively [Kakar *et al.*, 1992; Chi *et al.*, 1993; Tensen *et al.*, 1997; and chapter 2]. The sequence includes regions, which align from transmembrane domain (TM) V, to the intracellular carboxy-terminal tail, including intracellular and extracellular loops III as well as TMs VI and VII. Like the *X. laevis* pituitary receptor, the regions aligning to the putative hydrophobic transmembrane domains are highly conserved with the human GnRH receptor, with TM VI being the most conserved. The carboxy-terminal region of IL III is highly conserved including the alanine, which aligns to Ala^{261} of the human GnRH receptor, stressing the importance of this region in G-protein coupling [Myburgh *et al.*, 1998]. EL III of this receptor subtype is poorly conserved, with the highest amino acid identity (50%) to the *X. laevis* pituitary

receptor and a slightly lower identity to the corresponding region of the mammalian GnRH receptors (42-43%). The acidic amino acid, shown to be important for ligand selectivity (Glu^{301} in the mouse or Asp^{302} in the human GnRH receptor) in the mammalian GnRH receptors [Flanagan *et al.*, 1994] is not present. There is in fact, a proline residue in the corresponding position. The *X. laevis* pituitary receptor which does have a limited ligand selectivity (chapter 2), does have an acidic residue (Glu^{288}) in the corresponding position, but the preceding proline may alter the conformation of the loop so as to decrease the ligand selectivity (chapter 2). One could speculate, therefore, that the *Xl*b receptor subtype, which has two adjacent prolines in this position may have a reduced ligand specificity. This low specificity may be reflected by the relatively high IC_{50} for mGnRH and mGnRH analogues (table 3.3). The intracellular carboxy-terminal tail of this receptor has several putative phosphorylation sites, which may be important for desensitisation, which has been shown to be an integral part of cGnRH II stimulated M-current inhibition [Jones, 1987; for review, see Adams and Brown 1980].

Although I have shown by membrane binding assays, that a protein receptor is present in the sympathetic ganglia that binds cGnRH II with high affinity, I have been unable to clone the full-length receptor. In summary, however, one would expect this receptor to be a GPCR as G-protein coupling is required for M-current inhibition. The coupling mechanism is unknown, but does not seem to involve the G_q/G_{11} system typical of the other GnRH receptors. This receptor binds cGnRH II with a greater affinity than mGnRH and shows a rapid desensitisation upon stimulation with cGnRH II [Jones, 1987]. The endogenous ligand is unknown, but cGnRH II is a likely candidate as it reversibly inhibits M-current [Jones, 1987; for review, see Adams and Brown, 1980], was found in extracts of the sympathetic ganglia and shows specific binding to membranes from the ganglia.

From a molecular evolution perspective, as cGnRH II is highly conserved throughout evolution, occurring in all the major vertebrate groups, including cartilaginous fish, bony fish, amphibians, reptiles, birds, and several metatherian and early-evolved eutherian mammals [King *et al.*, 1989; King *et al.*, 1990; Dellovade *et al.*, 1993; King *et al.*, 1994b], as well as more recently, primates [Lescheid *et al.*, 1997] and humans [White *et al.*, 1998], it would be interesting to determine if this isoform of GnRH is used as a

neurotransmitter in these vertebrate species. The present study provides strong evidence for the involvement of endogenous cGnRH II in M-current suppression in the sympathetic ganglion of amphibian species via an as yet unidentified cGnRH II specific receptor.

Gonadotropin-releasing hormone receptor subtypes in the goldfish (*Carassius auratus*)

4.1 Summary

In the goldfish (*Carassius auratus*) the two endogenous forms of gonadotropin-releasing hormone, namely chicken GnRH II ([His^5,Trp^7,Tyr^8]GnRH) and salmon GnRH ([Trp^7,Leu^8]GnRH), stimulate the release of gonadotropins (GtHs) and growth hormone (GH) from the pituitary. This is thought to occur via the stimulation of two distinct receptors on the surface of gonadotropes and somatotropes. These receptors can be distinguished on the basis of differential GtH and GH releasing activities of endogenous GnRHs with variant amino acids in position 8 [Habibi *et al.*, 1992] as well as various analogues, which differentially stimulate or inhibit GnRH stimulated GH or GtH release [Murthy *et al.*, 1993; 1994; Murthy and Peter, 1994]. The cDNAs for two distinct GnRH receptor subtypes have been cloned from goldfish brain and pituitaries, designated GfA and GfB. These receptors have a 67% nucleotide homology and a 71% amino acid identity to each other. Both receptors have approximately 43% amino acid identity with the human GnRH receptor, while the GfB receptor subtype shows a distinctly higher homology to the catfish GnRH receptor (82%) than the GfA receptor (71%). Although neither receptor subtype shows identical pharmacological characteristics of either the GtH- or the GH-releasing receptors, they have a very distinct ligand specificity. Like the *X. laevis* pituitary GnRH receptor (chapter 2) and the catfish GnRH receptor [Tensen *et al.*, 1997], both receptor subtypes show a high specificity for cGnRH II in comparison to mGnRH and sGnRH. *In situ* hybridisation revealed that both receptor subtypes are expressed in the brain and pituitary. The GfA receptor is also expressed in the interstitial cells of the ovary and hepatocytes in the liver (Dr RE. Peter, University of Alberta, Edmonton, Canada). Thus both the GfA and GfB receptor subtypes may have important roles in pituitary regulation and both GH and GtH secretion, the GfA receptor may have an additional role due to its expression in non-regulatory tissues such as the liver.

4.2 Introduction

cGnRH II and sGnRH have been identified in the brains of the goldfish, *Carassius auratus* [Yu *et al.*, 1988; Kobayshi *et al.*, 1992; Kim *et al.*, 1995]. All teleosts examined to date have cGnRH II in combination with either the mammalian, salmon ([Trp^7,Leu^8]GnRH), catfish ([His^5,Asn^8]GnRH) or seabream GnRH ([Ser^8]GnRH) subtypes [for reviews, see Sherwood *et al.*, 1993, 1997; King and Millar 1997]. The distribution of the two endogenous forms of GnRH found in goldfish, has been studied using radioimmunoassays in combination with HPLC [Yu *et al.*, 1988; Kobayashi *et al.*, 1992] and immunocytochemistry [Kim *et al.*, 1995]. sGnRH predominated in the rostral brain areas including the olfactory bulb, telencephalon, hypothalamus and pituitary. cGnRH II, however, had a more widespread distribution, overlapping with that of sGnRH, but predominated in the posterior brain regions [Yu *et al.*, 1988; Kobayashi *et al.*, 1992; Kim *et al.*, 1995; for review, see Kim *et al.*, 1997]. Both sGnRH and cGnRH II may, therefore, be involved in regulating pituitary hormone secretion in goldfish.

Both the endogenous subtypes of GnRH in goldfish can stimulate the release of the gonadotropins (GtH II) and growth hormone (GH) [Marchant *et al.*, 1989; for review, see Trudeau, 1997]. GnRH has also been shown to stimulate GH secretion in the carp, *Cyprinus carpio* [Lin *et al.*, 1993], *Tilapia* hybrids [Melamed *et al.*, 1995] but not in catfish, *Clarias gariepinus* [Bosma *et al.*, 1997]. Most fish species have two forms of gonadotropins (GtHs) [Kawauchi *et al.*, 1989], referred to as GtH I and GtH II, but due to the similarities of these GtHs with mammalian FSH and LH, they are often referred to as fish FSH and LH [Prat *et al.*, 1996].

GnRH receptors have been detected on the surface of gonadotropes and somatotropes in the goldfish pituitary, using electron microscopy [Cook *et al.*, 1991]. Binding studies on goldfish pituitaries have suggested the presence of two binding sites, distinguished by their binding dynamics, as a high affinity, low capacity site and a low affinity, high capacity site [Habibi *et al.*, 1987]. The potency of both GtH and GH release is, however, consistent with the high affinity

binding sites of the pituitary [Habibi *et al.*, 1989]. Both sGnRH and cGnRH II can displace a ^{125}I-labelled sGnRH analogue from goldfish pituitary membrane preparations [Habibi *et al.*, 1987]. Furthermore, both the endogenous peptides can displace biotinylated sGnRH analogue from the surfaces of both somatotropes and gonadotropes in the pituitary [Cook *et al.*, 1991]. It was originally suggested that the two endogenous peptides bind the same receptor on the surface of somatotropes and gonadotropes, but they could activate two different intracellular signalling systems in the different cells, stimulating the release of GH from somatotropes and GtHs from gonadotropes [Chang *et al.*, 1991(a); 1991(b)]. Despite these binding results, the receptors on the two cell types are likely to be different as the GtH II- and GH-releasing activity of different GnRH analogues differ substantially [Habibi *et al.*, 1992; Murthy *et al.*, 1994; Murthy and Peter, 1994]. For GtH II-releasing activity cGnRH II is more potent than sGnRH and mGnRH. For GH-releasing activity, however, sGnRH is more potent than mGnRH and cGnRH II, which are approximately equipotent [Habibi *et al.*, 1992]. The GH-releasing activity also appears to be more selective for GnRH analogues with variations in position 8, such as [His^{8}]GnRH, [Leu^{8}]GnRH and [Tyr^{8}]GnRH, while the GtH II-releasing activity shows a lower response to these analogues [Habibi *et al.*, 1992]. The two receptors can also be distinguished by their response to the GnRH antagonists. Analogue E ([Ac-Pro^{1},D-Fpa^{2},D-Trp^{3,6}]GnRH) is pure antagonist for both salmon and cGnRH II stimulated GtH II and GH release, while analogue C ([Ac-Pro^{1}, D-Fpa^{2}, D-Trp^{3,6},Trp^{7},Leu^{8}]GnRH) inhibits sGnRH and cGnRH II stimulated GtH-II release, but stimulates GH release. A third analogue, analogue L ([Ac-D-Nal^{1}, D-Cpa^{2}, D-Pal^{3},Arg^{5},D-Pal^{6},D-Ala^{10}]GnRH) weakly stimulates GtH II release but inhibits GH release [Murthy and Peter, 1994; Murthy *et al.*, 1993; 1994].

The possibility of two GnRH receptor subtypes in the goldfish pituitary was, therefore, investigated. Two receptor subtypes were identified from goldfish pituitary and brain. These receptor subtypes show a low homology with the mammalian GnRH receptors and one receptor shows a high homology with the catfish GnRH receptor [Tensen *et al.*, 1997]. Pharmacological studies of the two

receptors, transiently transfected into COS-1 cells revealed distinct functional characteristics between the receptors, which may correspond to the functional activity of the receptors.

4.3 Materials and methods

4.3.1 Peptides

The following peptides were used in this study, the agonists: GnRH; [His^5,Trp^7,Tyr^8]GnRH (Chicken GnRH II, cGnRH II); [Gln^8]GnRH (Chicken GnRH I, cGnRH I); [Tyr^8]GnRH; [D-Arg^6,Trp^7,Tyr^8]GnRH; and [D-Arg^6,Trp^7,Leu^8]GnRH were prepared by solid-phase synthesis and purified by C-18 reverse-phase chromatography. [Ser^8]GnRH (seabream GnRH, sbGnRH); [Trp^7,Leu^8]GnRH (salmon GnRH, sGnRH); [His^5,D-Arg^6,Leu^8]GnRH; [His^8]GnRH was from R.W. Roeske and the antagonists: [Ac-Pro^1,D-Fpa^2,D-Trp^{3,6}]GnRH (analogue E); [Ac-Pro^1, D-Fpa^2, D-Trp^{3,6},Trp^7,Leu^8]GnRH (analogue C); and [Ac-D-Nal^1, D-Cpa^2, D-Pal^3,Arg^5,D-Pal^6,D-Ala^{10}]GnRH (analogue L) were gifts from J. Rivier.

4.3.2 Experimental animals and tissue preparation

Goldfish material was supplied by Dr R.E. Peter (University of Alberta, Edmonton, Canada). Female and male goldfish (30-40 g body weight) were purchased from Grassyfork Fisheries (Martinsville Indiana). The fish were maintained in a 300-litre flow through aquarium at 15°C under 16 h light and 8 h dark photoperiod and were fed daily, *ad libitum*, with commercially prepared fish pellets. Goldfish were anaesthetised and livers, whole brain and pituitaries were dissected. Dissected material was immediately frozen on dry ice and stored at -70°C until needed. Genomic DNA was purified from five livers as described in section 2.3.1. Total RNA was purified from whole brain and pituitaries by extraction with guanidium thiocynate [Chomczynski and Sacchi, 1987].

4.3.3 Cloning of goldfish GnRH receptors

Degenerate PCR primers, JH5s and JH6a$_2$, (see section, 2.3.3 for primer sequence) designed to conserved regions of the mammalian GnRH receptors, were used to amplify two putative GnRH receptor subtypes from goldfish genomic DNA (GfA and GfB) (see section, 2.3.3 - 2.3.5 for a detailed description of methods). The nucleotide sequence of these clones was used to design gene-specific primers to amplify up the remainder of these GnRH receptor sequences from cDNA (see appendices, 7.11 and 7.12 for primer nomenclature, sequence and location).

4.3.4 cDNA synthesis and amplification

This work was conducted in collaboration with Prof. N. Illing (University of Cape Town, Biochemistry Department), but all the methods used are described here, for completeness. The work on pituitary cDNA synthesis, amplification, analysis, cloning and sequencing of both receptor subtypes was conducted by Prof. Illing. I synthesised the whole brain cDNA, but PCR amplification was done by Prof. Illing. I did all subsequent product analysis, cloning and characterisation.

cDNA was synthesised from total RNA purified from goldfish whole brains and pituitaries, using the Marathon cDNA synthesis kit (Clonetech). [α-^{32}P]dCTP (1 μCi/μl, Amersham) was added to the cDNA synthesis reaction in order to enable the quantification of cDNA synthesis. Double stranded cDNA was analysed by agarose gel electrophoresis, followed by autoradiography. Adaptor primers, were ligated onto the 5' ends of the double stranded cDNA.

The 5' end of GfA cDNA was amplified using nested PCR with GfA.1as and GfA.3as (see appendix 7.11 for location and sequence) in combination with the adaptor primers AP1 and AP2 (Clonetech). PCR products were then analysed by Southern blot using the nested primer GfA.2s end-labelled with fluorescene-11-dUTP using the 3' Oligonucleotide labelling and detection system (ECL, Amersham). A single product of 2800 bp was detected, which was cloned into the pGEM-T vector (Promega, see appendix 7.13 for map). Nucleotide sequence

analysis of this PCR product, showed that it contained the start codon for the GnRH receptor. The 3' end of the GnRH receptor, extending from extracellular loop 3 to the C-terminal tail, was similarly cloned, by nested PCR amplification of goldfish pituitary cDNA with GfA.2s and GfA.4s (see appendix 7.11), in combination with AP1 and AP2. Several PCR products were identified on a Southern blot, which was probed using the ECL system (Amersham) and fluorescene labelled GfA.1as primer. One of these products contained the nucleotide sequence of the GfA receptor extending to a stop codon in the C-terminal tail. This nucleotide sequence information was used to design nested gene-specific primers to the non-coding 5' and 3' regions of the GfA receptor. The primers were as follows:

GfA.5s 5' ctcactagtgcgttatagaggtgcaga 3'
GfA.6s 5' atctggataatcaggaatttcttg 3'
GfA.7as 5' tagttcgaaccatcgcttcatcgt 3'
GfA.8as 5' ctcagagtctggttcattatggag 3'

The nested primers, GfA.5s and GfA.6s to the 5' untranslated region were used in combination with the nested 3' primers, GfA.7as and GfA.8as to amplify up the full-length receptor from goldfish pituitary cDNA. The nucleotide sequence of several clones, amplified using both Klentaq (Clonetech) and Pfu DNA polymerases (Stratagene) was determined using both manual (T7 sequenase, Amersham) and automated sequencing.

The 5' end of the GfB receptor was cloned by PCR amplification on Marathon pituitary cDNA with primers GfB.1as and GfB.3as in combination with the AP1 and AP2 primers (see appendix 7.12 for primer sequence and location). Multiple products were identified in Southern blot using the ECL system (Amersham) on the PCR reaction with the nested fluorescene end-labelled GfB.2s primer. These products were cloned into the pGEM-T vector. No clones with a full-length 5' open reading frame were identified. Whole brain cDNA was therefore, synthesised using the Marathon cDNA synthesis kit (Clonetech) and was amplified as above. Sequence analysis of the PCR products subcloned into the

pGEM-T vector showed that one of the clones contained the full-length 5' coding region of the GfB receptor. This nucleotide sequence information was used to design two primers to the 5' untranslated region. The primers were as follows:

GfB.4s 5' cgagtggagcatgtctagatgctaata 3'

GfB.5s 5' actgtccgcatattagtggaaatgagg 3'

The nested primers, GfB.4s and GfB.5s were used in combination with the AP1 and AP2 primers, to amplify up the full-length type B receptor from Marathon goldfish brain cDNA. The PCR product was subcloned and the nucleotide sequence of 4 clones, determined.

4.3.5 Transient transfection of COS-1 cells

The full-length Type A and Type B goldfish GnRH receptors were subcloned into pcDNA I/Amp (Invitrogen), a mammalian expression vector containing the SV40 early promoter. DNA for transfection was prepared from a 500 ml overnight culture of 2 x YT broth (10 g/L NaCL, 10 g/L Yeast extract, 16 g/L Bactotryptone) containing ampicillin (100 μg/ml). See section 2.3.10 (chapter 2) for a detailed description of transfection methods.

4.3.6 Inositol Phosphate Assay

Inositol phosphate assays were as described in chapter 2, section 2.3.12.

4.4 Results

4.4.1 The primary structure

Two full-length putative goldfish GnRH receptor cDNA subtypes, GfA and GfB, were identified after PCR (fig. 4.1A and B). Start codons were assigned on the basis of homology to the cloned GnRH receptors [for review, see Sealfon *et al.*, 1997]. Both cDNA clones encoded putative GnRH receptors with seven hydrophobic transmembrane domains and like the *Xenopus laevis* pituitary GnRH receptor (chapter 2) and the catfish GnRH receptor, [Tensen *et al.*, 1997], intracellular carboxy-terminal tails. Eight full-length clones of GfA were sequenced. Two differences in the amino acid sequence occurred of which the

consensus sequence of the eight clones was taken to be wildtype. A C to T transition in transmembrane helix III (TM III) resulting in Ala^{125} being changed to a Val, and a C to T transition in extracellular loop II resulting in His^{195} being changed to a Tyr. All clones, however, gave a similar inositol phosphate response. Both Klentaq (Clonetech) and Pfu (Stratagene) DNA polymerases were used to amplify these clones, and both polymerases have a high fidelity resulting from the 3'/5' exonuclease activity [Barnes, 1994]. The differences in nucleotide sequence may, therefore, have been due to either gene polymorphisms or as a result of the tetraploidy that exists in goldfish. Four full-length clones of GfB were sequenced. The coding regions of these all resulted in identical amino acid receptors, although, differences in the nucleotide sequences occurred in both the coding and non-coding regions.

GfB and GfA have a 67% nucleotide sequence homology and a 71% amino acid identity, with highest homology in the transmembrane domains (fig. 4.2, table 4.1). GfB, however, has a higher homology to the catfish GnRH receptor than does the GfA receptor, with percent amino acid identities of 82% and 71% respectively (fig. 4.3, table 4.1). The identities of the GfA and GfB receptors, to the primary sequence of the human GnRH receptor, were both approximately 43% (table 4.1). Hydrophobicity plots of the amino acid sequences of these receptors, clearly show the seven putative hydrophobic transmembrane domains (fig. 4.3). They also show a hydrophobic region towards the carboxy terminal end of the cytoplasmic tail.

4.4.2 Pharmacological characterisation of goldfish GnRH receptors.

Both the GfA and GfB GnRH receptor subtypes coupled to phospholipase C when transiently transfected into COS-1 cells as revealed by the stimulation of inositol phosphates (IPs) by GnRH agonists (fig. 4.4, table 4.2). Both the GfA and GfB receptors showed the same order of potencies for natural GnRH with cGnRH II > sGnRH > mGnRH > sbGnRH (fig. 4.4). However, the ED_{50}s, of the endogenous GnRH agonists for the two receptor subtypes, however, differed substantially (table 4.2). GfB seems to have a lower potency than the GfA receptor with all the

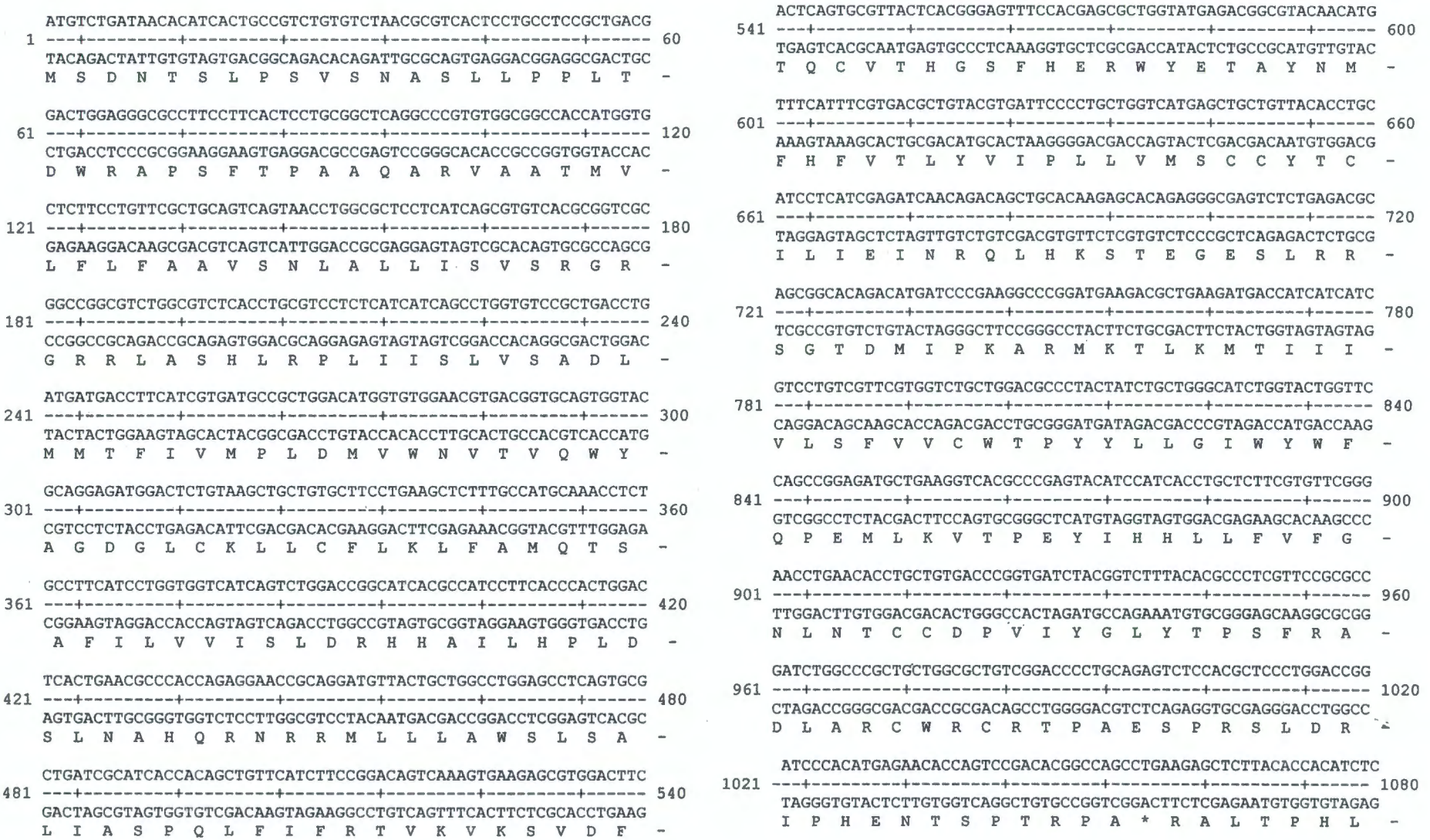
GnRH agonists tested. This may be due to a lower affinity or a lower expression of the GfB receptor in the transfected cells. The relative increase in ED_{50}s are, however, not proportional and thus indicates a different ligand selectivity of the two receptors (table 4.2). The relative potency for mGnRH for the GfA and GfB receptors compared to sGnRH, was equal (0.02), while cGnRH II was much more potent in stimulating IP production in GfA than GfB, with relative potencies to sGnRH, of 153 and 16.5 respectively. The GfB receptor is also much more selective to GnRH agonists with variant amino acids in position 8, as while having a similar potency to mGnRH as the GfA receptor, it has a much lower potency for [His^8]GnRH, sbGnRH, and [Tyr^8]GnRH than the GfA receptor (table 4.2).

The analogues of GnRH containing a D-Arg in position 6 had variable effects on the potencies. The D-Arg^6 sGnRH showed a 10-fold higher potency in the GfA receptor, but only a 2-fold higher potency in the GfB receptor (table 4.2). [His^5,D-Arg^6,Leu^8]GnRH had an approximately equal potency to sGnRH in the GfA receptor, while it had a greater than 10-fold decrease in the GfB receptor compared to sGnRH. [D-Arg^6,Trp^7,Tyr^8]GnRH had an equal potency to cGnRH II in the GfA receptor and an approximately 4-fold higher potency than cGnRH II in the Gf E receptor.

The analogues E, C and L, which have different stimulatory and inhibitory properties on GtH II and GH secretion, were also tested. All three analogues appeared to behave as antagonists, for both sGnRH (fig. 4.5) and cGnRH II (data not shown) stimulated inositol phosphate production.

Figure 4.1. Nucleotide and deduced amino acid sequences of the GfA (**A**) and GfB (**B**) receptors cloned form pituitary and whole brain cDNA.

Figure 4.1.A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M S D N T S L P S V S N A S L L P P L T -
GACTGGAGGGCGCCTTCCTTCACTCCTGCGGCTCAGGCCCGTGTGGCGGCCACCATGGTG
CTGACCTCCCGCGGAAGGAAGTGAGGACGCCGAGTCCGGGCACACCGCCGGTGGTACCAC
D W R A P S F T P A A Q A R V A A T M V -
CTCTTCCTGTTCGCTGCAGTCAGTAACCTGGCGCTCCTCATCAGCGTGTCACGCGGTCGC
GAGAAGGACAAGCGACGTCAGTCATTGGACCGCGAGGAGTAGTCGCACAGTGCGCCAGCG
L F L F A A V S N L A L L I S V S R G R -
GGCCGGCGTCTGGCGTCTCACCTGCGTCCTCTCATCATCAGCCTGGTGTCCGCTGACCTG
CCGGCCGCAGACCGCAGAGTGGACGCAGGAGAGTAGTAGTCGGACCACAGGCGACTGGAC
G R R L A S H L R P L I I S L V S A D L -
ATGATGACCTTCATCGTGATGCCGCTGGACATGGTGTGGAACGTGACGGTGCAGTGGTAC
TACTACTGGAAGTAGCACTACGGCGACCTGTACCACACCTTGCACTGCCACGTCACCATG
M M T F I V M P L D M V W N V T V Q W Y -
GCAGGAGATGGACTCTGTAAGCTGCTGTGCTTCCTGAAGCTCTTTGCCATGCAAACCTCT
CGTCCTCTACCTGAGACATTCGACGACACGAAGGACTTCGAGAAACGGTACGTTTGGAGA
A G D G L C K L L C F L K L F A M Q T S -
GCCTTCATCCTGGTGGTCATCAGTCTGGACCGGCATCACGCCATCCTTCACCCACTGGAC
CGGAAGTAGGACCACCAGTAGTCAGACCTGGCCGTAGTGCGGTAGGAAGTGGGTGACCTG
A F I L V V I S L D R H H A I L H P L D -
TCACTGAACGCCCACCAGAGGAACCGCAGGATGTTACTGCTGGCCTGGAGCCTCAGTGCG
AGTGACTTGCGGGTGGTCTCCTTGGCGTCCTACAATGACGACCGGACCTCGGAGTCACGC
S L N A H Q R N R R M L L L A W S L S A -
CTGATCGCATCACCACAGCTGTTCATCTTCCGGACAGTCAAAGTGAAGAGCGTGGACTTC
GACTAGCGTAGTGGTGTCGACAAGTAGAAGGCCTGTCAGTTTCACTTCTCGCACCTGAAG
L I A S P Q L F I F R T V K V K S V D F -
ACTCAGTGCGTTACTCACGGGAGTTTCCACGAGCGCTGGTATGAGACGGCGTACAACATG
TGAGTCACGCAATGAGTGCCCTCAAAGGTGCTCGCGACCATACTCTGCCGCATGTTGTAC
T Q C V T H G S F H E R W Y E T A Y N M -
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AAAGTAAAGCACTGCGACATGCACTAAGGGGACGACCAGTACTCGACGACAATGTGGACG
F H F V T L Y V I P L L V M S C C Y T C -
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I L I E I N R Q L H K S T E G E S L R R -
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S G T D M I P K A R M K T L K M T I I I -
GTCCTGTCGTTCGTGGTCTGCTGGACGCCCTACTATCTGCTGGGCATCTGGTACTGGTTC
CAGGACAGCAAGCACCAGACGACCTGCGGGATGATAGACGACCCGTAGACCATGACCAAG
V L S F V V C W T P Y Y L L G I W Y W F -
CAGCCGGAGATGCTGAAGGTCACGCCCGAGTACATCCATCACCTGCTCTTCGTGTTCGGG
GTCGGCCTCTACGACTTCCAGTGCGGGCTCATGTAGGTAGTGGACGAGAAGCACAAGCCC
Q P E M L K V T P E Y I H H L L F V F G -
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N L N T C C D P V I Y G L Y T P S F R A -
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D L A R C W R C R T P A E S P R S L D R -
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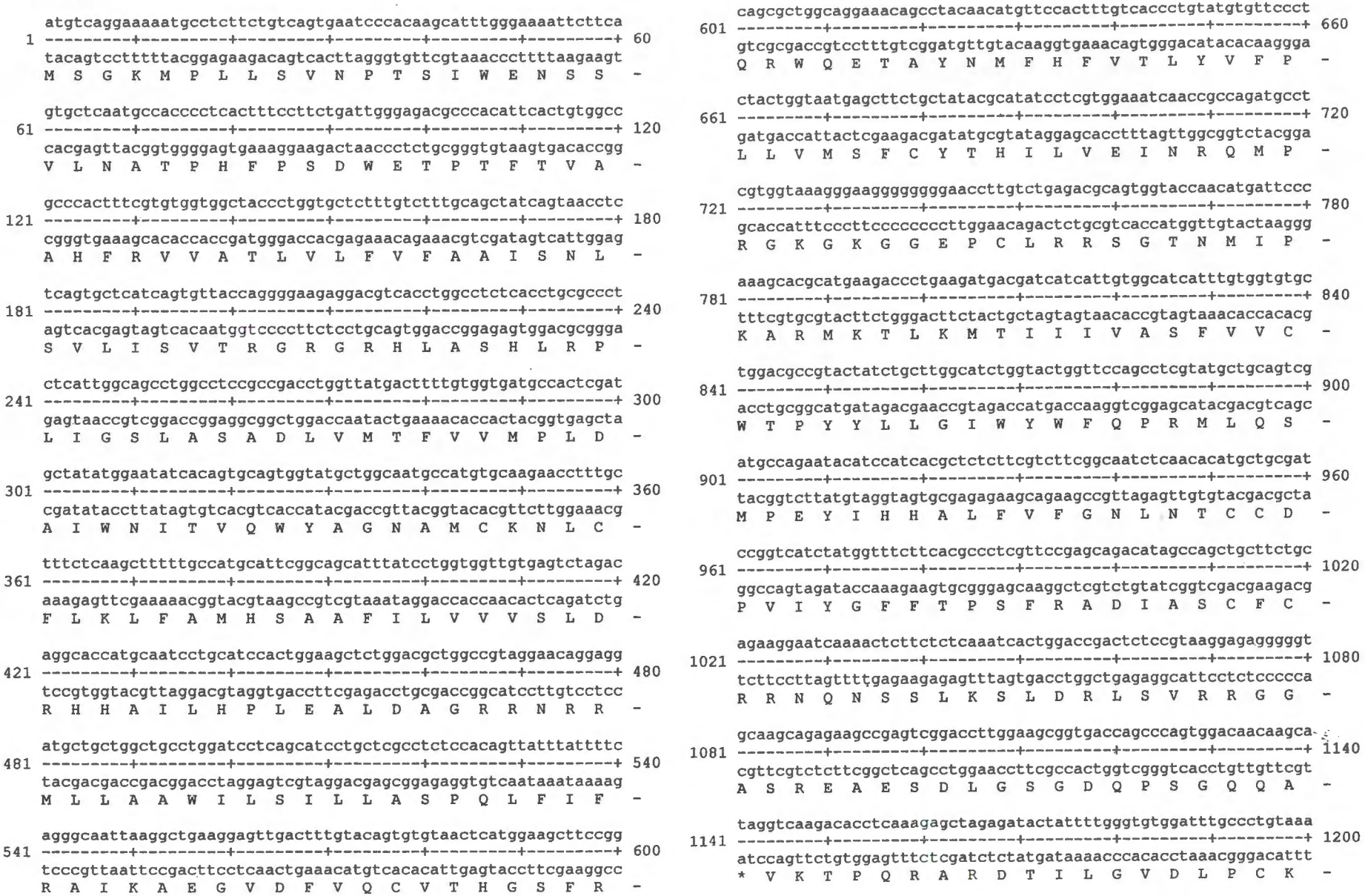
Figure 4.1.B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M S G K M P L L S V N P T S I W E N S S -
gtgctcaatgccacccctcactttccttctgattgggagacgcccacattcactgtggcc
61 120
cacgagttacggtggggagtgaaaggaagactaaccctctgcgggtgtaagtgacaccgg
V L N A T P H F P S D W E T P T F T V A -
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121 180
cgggtgaaagcacaccaccgatgggaccacgagaaacagaaacgtcgatagtcattggag
A H F R V V A T L V L F V F A A I S N L -
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181 240
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S V L I S V T R G R G R H L A S H L R P -
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241 300
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L I G S L A S A D L V M T F V V M P L D -
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421 480
tccgtggtacgttaggacgtaggtgaccttcgagacctgcgaccggcatccttgtcctcc
R H H A I L H P L E A L D A G R R N R R -
atgctgctggctgcctggatcctcagcatcctgctcgcctctccacagttatttatttc
481 540
tacgacgaccgacggacctaggagtcgtaggacgagcggagaggtgtcaataaataaaag
M L L A A W I L S I L L A S P Q L F I F -
agggcaattaaggctgaaggagttgactttgtacagtgtgtaactcatggaagcttccgg
541 600
tcccgttaattccgacttcctcaactgaaacatgtcacacattgagtaccttcgaaggcc
R A I K A E G V D F V Q C V T H G S F R -
cagcgctggcaggaaacagcctacaacatgttccactttgtcaccctgtatgtgttccct
601 660
gtcgcgaccgtcctttgtcggatgttgtacaaggtgaaacagtgggacatacacaaggga
Q R W Q E T A Y N M F H F V T L Y V F P -
ctactggtaatgagcttctgctatacgcatatcctcgtggaaatcaaccgccagatgcct
661 720
gatgaccattactcgaagacgatatgcgtataggagcacctttagttggcggtctacgga
L L V M S F C Y T H I L V E I N R Q M P -
cgtggtaaagggaaggggggggaaccttgtctgagacgcagtggtaccaacatgattccc
721 780
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R G K G K G G E P C L R R S G T N M I P -
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781 840
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K A R M K T L K M T I I I V A S F V V C -
tggacgccgtactatctgcttggcatctggtactggttccagcctcgtatgctgcagtcg
841 900
acctgcggcatgatagacgaaccgtagaccatgaccaaggtcggagcatacgacgtcagc
W T P Y Y L L G I W Y W F Q P R M L Q S -
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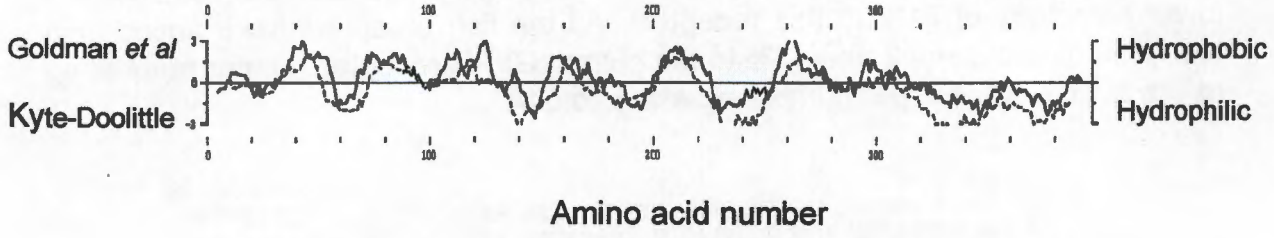
901 960
tacggtcttatgtaggtagtgcgagagaagcagaagccgttagagttgtgtacgacgcta
M P E Y I H H A L F V F G N L N T C C D -
ccggtcatctatggtttcttcacgccctcgttccgagcagacatagccagctgcttctgc
961 1020
ggccagtagataccaaagaagtgcgggagcaaggctcgtctgtatcggtcgacgaagacg
P V I Y G F F T P S F R A D I A S C F C -
agaaggaatcaaaactcttctctcaaatcactggaccgactctccgtaaggagaggggt
1021 1080
tcttccttagttttgagaagagagtttagtgacctggctgagaggcattcctctccccca
R R N Q N S S L K S L D R L S V R R G G -
gcaagcagagaagccgagtcggaccttggaagcggtgaccagcccagtggacaacaagca
1081 1140
cgttcgtctcttcggctcagcctggaaccttcgccactggtcgggtcacctgttgttcgt
A S R E A E S D L G S G D Q P S G Q Q A -
taggtcaagacacctcaaagagctagagatactattttgggtgtggatttgccctgtaaa
1141 1200
atccagttctgtggagtttctcgatctctatgataaaacccacacctaaacgggacattt
* V K T P Q R A R D T I L G V D L P C K -

Figure 4.2. Sequence alignment of the goldfish GnRH receptor subtypes A and B to the human and catfish GnRH receptors. Alignments were done using the Gap program of GCG. The putative transmembrane domains are indicated (Donnelly *et al.*, 1994). cf, catfish; GfB, goldfish B; GfA, goldfish A; h, human. The two goldfish receptor subtypes A and B have a 71% amino acid identity, while GfB has a 81% amino acid identity to the cfGnRH receptor, and the GfA subtype shows a lower homology of 71% to this receptor. All the fish receptors have amino acid identities of between 42 and 43% to the human GnRH receptor. Upper numbering refers to GfB, while lower numbering refers to GfA.

```
          1                                                          |---------- 49
 hGnRHR   -----MANSA SPEQNQNHCS AINNSI..PL MQGNLPTLTL SGKIRVTVTF
cfGnRHR   MSGNTTLLLS NPTNVLDNSS VLNVSVSPPV LKWETPTFTT AARFRVAATL
    GfB   MSGKMPLLSV NPTSIWENSS VLNATPHFP. SDWETPTFTV AAHFRVVATL
    GfA   ---------- -MSDNTSLPS VSNASLLPPL TDWRAPSFTP AAQARVAATM
                     1                                                    39

         50------TM I---------------|          |----------TM II--95
 hGnRHR   FLFLLSATFN ASFLLKLQKW TQKKEKGKKL SRMKLLLKHL TLANLLETLI
cfGnRHR   VLFVFRAASN LSVLLSVTRG RGR....RLA SHLRPLIASL ASADLVMTFV
    GfB   VLFVFAAISN LSVLISVTRG RGR....HLA SHLRPLIGSL ASADLVMTFV
    GfA   VLFLFAAVSN LALLISVSRG RGR....RLA SHLRPLIISL VSADLMMTFI
         40                                                         85

         96-------------|           |------------TM III------------| 145
 hGnRHR   VMPLDGMWNI TVQWYAGELL CKVLSYLKLF SMYAPAFMMV VISLDRSLAI
cfGnRHR   VMPLDAVWNV TVQWYAGDAM CKLMCFLKLF AMHSAAFILV VVSLDRHHAI
    GfB   VMPLDAIWNI TVQWYAGNAM CKNLCFLKLF AMHSAAFILV VVSLDRHHAI
    GfA   VMPLDMVWNV TVQWYAGDGL CKLLCFLKLF AMQTSAFILV VISLDRHHAI
         86                                                         135

        146       |-----------TM IV------------|                    190
 hGnRHR   TRPLALKSNS KVGQSMVGLA WILSSVFAGP QLYIFRMIHL ADSSGQTKVF
cfGnRHR   LHPLDTLDAG RRNRRMLLTA .ILSLLLASP QLFIFRAIKA KGVD.....F
    GfB   LHPLEALDAG RRNRRMLLAA WILSILLASP QLFIFRAIKA EGVD.....F
    GfA   LHPLDSLNAH QRNRRMLLLA WSLSALIASP QLFIFRTVKV KSVD.....F
         136                                                        180

         191                |---------------TM V---------------|    240
 hGnRHR   SQCVTHCSFS QWWHQAFYNF FTFSCLFIIP LFIMLICNAK IIFTLTRVLH
cfGnRHR   VQCATHGSFQ QHWQETAYNM FHFVTLYVFP LL.MSLCYTR ILVEINRQMH
    GfB   VQCVTHGSFR QRWQETAYNM FHFVTLYVFP LLVMSFCYTH ILVEINRQMP
    GfA   TQCVTHGSFH ERWYETAYNM FHFVTLYVIP LLVMSCCYTC ILIEINRQLH
         181                                                        230

         241                          |---------------TM VI-------290
 hGnRHR   QDPH...ELQ LNQSKNN.IP RARLKTLKMT VAFATSFTVC WTPYYVLGIW
cfGnRHR   RSKDKAGEPC LRRSGTDMIP KARMKTLKMT IIIVASFVIC WTPYYLLGIW
    GfB   RGKGKGGEPC LRRSGTNMIP KARMKTLKMT IIIVASFVVC WTPYYLLGIW
    GfA   KSTE..GE.S LRRSGTDMIP KARMKTLKMT IIIVLSFVVC WTPYYLLGIW
         231                                                        277

         291----|         |------------TM VII------------|          340
 hGnRHR   YWFDPEMLNR LSDPVNHFFF LFAFLNPCFD PLIYGYFSL
cfGnRHR   YWFQPQMLHV IPDYVHHVFF VFGNLNTCCD PVIYGFFTPS FRADLSRCFC
    GfB   YWFQPRMLQS MPEYIHHALF VFGNLNTCCD PVIYGFFTPS FRADIASCFC
    GfA   YWFQPEMLKV TPEYIHHLLF VFGNLNTCCD PVIYGLYTPS FRADLARCWR
         278                                                        327

         341                                                  380
cfGnRHR   WRNQNASAKS LPHFSGHRRE VSGEAESDLG SGDQ.SGQ
    GfB   RRNQNSSLKS LDRLSVRRGG ASREAESDLG SGDQPSGQQA
    GfA   CRTPAESPRS LDRIPHENTS PTRPA
         328                          352
```

A: GfA



B: GfB

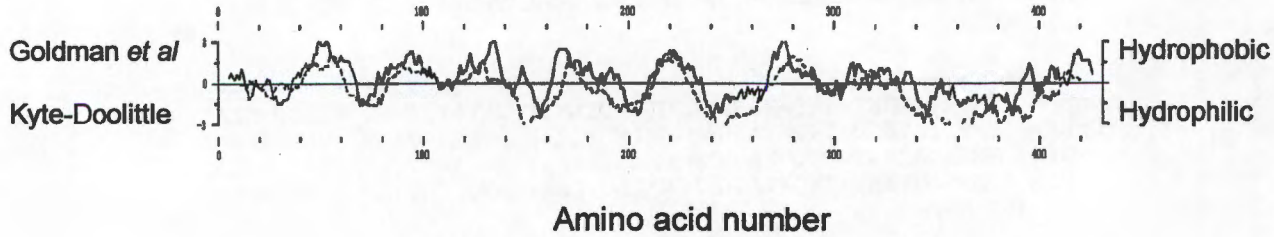


Figure 4.3. Hydrophobicity plots of the GfA (**A**) and GfB (**B**) receptor subtypes, showing the characteristic seven hydrophobic transmembrane domains. Hydrophobicity plots were done using the Wisconsin Package Version 9.0, Genetics Computer Group (GCG) Madison, Wisconsin (Pepstructure).

Table 4.1. Percent amino acid identity of the human, catfish, GFA and GFB GnRH receptors.

Human	42	43.4	43
Catfish		71	82
GfA			71
GfB			

Table 4.2. ED_{50}s of GnRH agonists for inositol phosphate production in COS-1 cells transiently transfected with the Goldfish A and B GnRH receptor subtypes. Data are calculated as the mean of three separate experiments.

Peptide (common name)	GfA GnRH-R		GfB GnRH-R	
	ED_{50} (nM)	Relative Potency to Salmon GnRH	ED_{50} (nM)	Relative Potency to Salmon GnRH
[Trp^7, Leu^8] GnRH (salmon GnRH)	4.6±0.3	1	56±20	1
[His^5, Trp^7, Tyr^8]GnRH (chicken GnRH II)	0.03±0.01	153	3.4±2	16.5
Mammalian GnRH	210±69	0.02	2850±21	0.02
[Ser^8]GnRH (seabream GnRH)	684±100	0.007	>10 000	>0.006
[His^8]GnRH	11±3	0.42	>20 000	>0.003
[Tyr^8]GnRH	4.65±1	1	640±100	0.08
[D-Arg^6, Trp^7, Tyr^8]GnRH	0.03±0.01	153	0.8±0.5	70
[His^5, D-Arg^6, Leu^8]GnRH	3.3±2	1.4	630±100	0.08
[D-Arg^6, Trp^7, Leu^8]GnRH	0.4±0.2	11.5	21.7±10	2.6

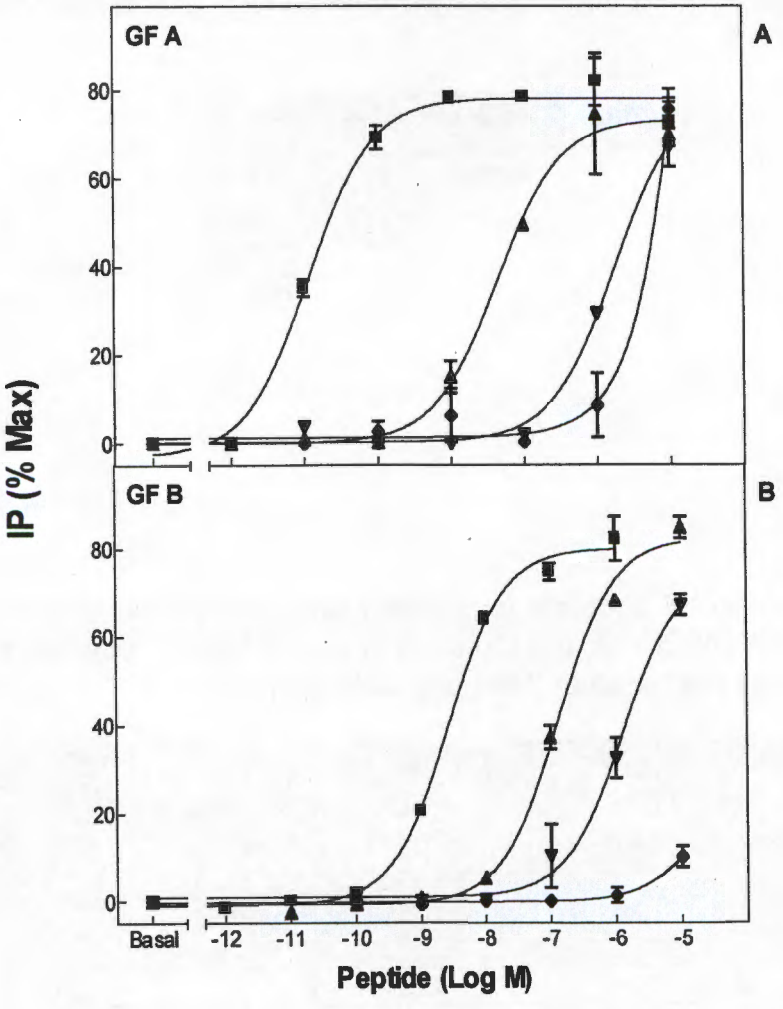


Figure 4.4. Inositol phosphate production in COS-1 cells transiently transfected with the GFA (**A**) and GF E (**B**) GnRH receptor subtypes, after stumulation for 1 h with various GnRH agonists. (▼) mammalian GnRH; (▲) [Trp^7,Leu^8]GnRH (salmon GnRH); (■) [His^5,Trp^7,Tyr^8]GnRH (chicken GnRH II) and (◆) [Ser^8]GnRH (seabream GnRH). For both receptor subtypes, chicken GnRH II has the highest potency, followed by salmon GnRH, mammalian GnRH and seabream GnRH respectively.

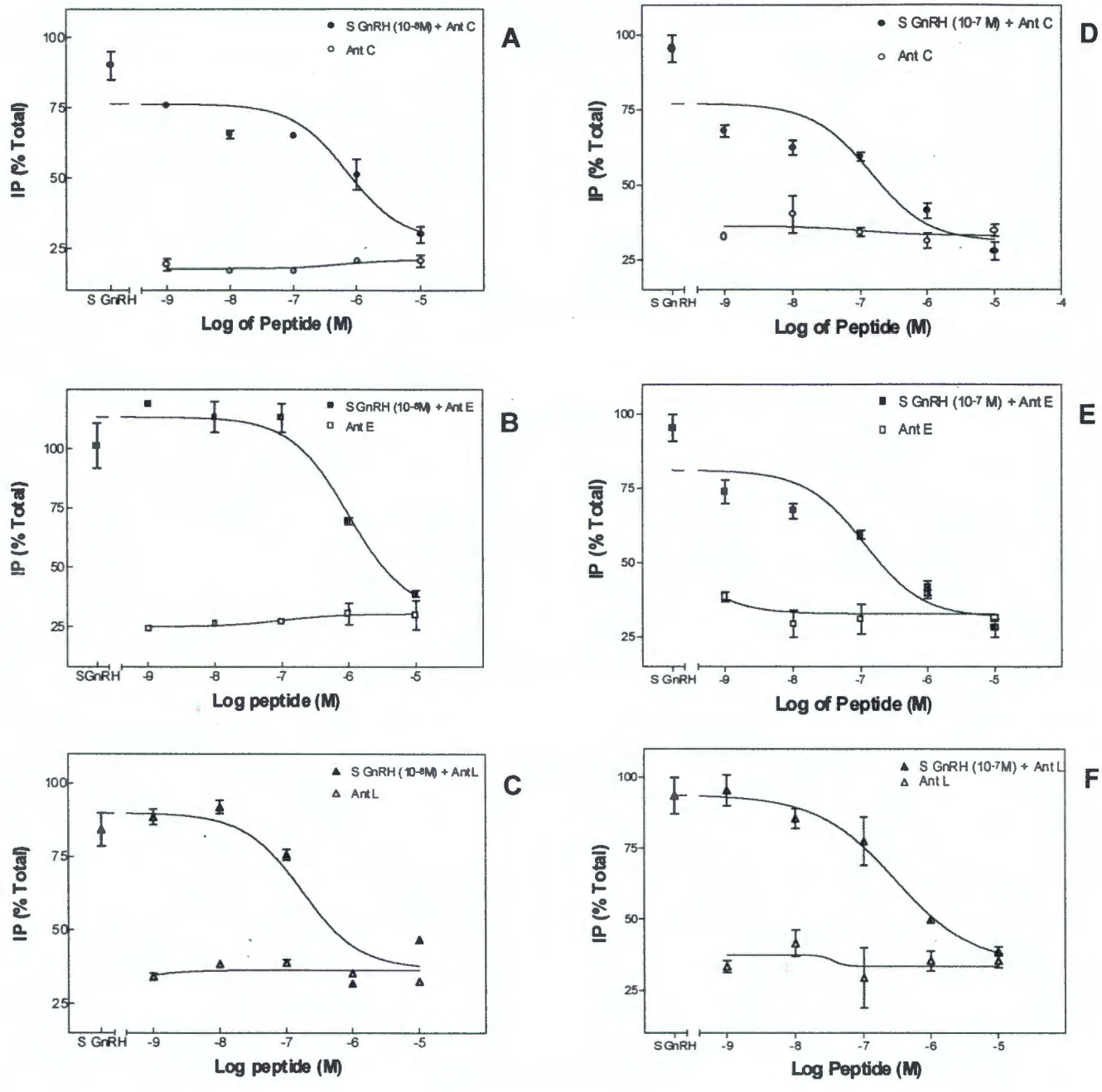


Figure 4.5. Inhibition of inositol phosphate production in COS-1 cells transfected with the GfA (A-C), and the GfB (D-F) GnRH receptor subtypes stimulated with salmon GnRH at 10^{-8} M in the presence of increasing concentrations of analogues C, E and L. **A**: (●) salmon GnRH with [Ac-Pro^{1}, D-Fpa^{2}, D-Trp^{3,6},Trp^{7},Leu^{8}]GnRH (analogue C), and (○) analogue C alone; **B**: (■) salmon GnRH with [Ac-Pro^{1},D-Fpa^{2},D-Trp^{3,6}]GnRH (analogue E), and (□) analogue E alone; **C**: (▲) salmon GnRH with [Ac-D-Nal^{1}, D-Cpa^{2}, D-Pal^{3},Arg^{5},D-Pal^{6},D-Ala^{10}]GnRH (analogue L), and (△) analogue L alone.

4.5 **Discussion**

The dual effect of GnRHs in the goldfish (*Carassius auratus*) on gonadotropes and somatotropes described by Marchant and co-workers [1989] has initiated the investigation of the receptor subtypes by a detailed analysis of the pharmacological response of the two cell types to different GnRH agonists and antagonists [Habibi *et al.*, 1992; Murthy and Peter, 1994; Murthy *et al.*, 1994; 1993]. On the basis of *in vivo* studies, there is evidence for two distinct receptor subtypes in the goldfish, which mediate the synthesis and release of GtH II and GH from gonadotropes and somatotropes, respectively. In this chapter the occurrence of two receptor subtypes in goldfish, was investigated using sequence information from the cloned mammalian GnRH receptors. The pharmacology of these two receptor subtypes was analysed with regard to previous information on the two putative receptors in the goldfish pituitary.

Amplification of genomic DNA with degenerate oligonucleotide primers designed to conserved regions in the cloned mammalian GnRH receptors revealed two putative GnRH receptor subtypes in the goldfish, designated GfA and GfB. The complete cDNAs encoding these receptors were cloned from pituitary and brain, respectively. These receptors, like the other cloned non-mammalian receptors showed a low amino acid identity to the mammalian GnRH receptors (approximately 43%). The two goldfish receptor subtypes had a relatively high amino acid identity to each other (71%), but the GfB receptor was more similar to the catfish GnRH receptor [Tensen *et al.*, 1997] (82% identity) compared to the GfA receptor (71%). These receptor subtypes are not duplicate genes, which result from tetraploidisation, as the homology in the coding region of duplicate genes would be greater than 90% [Lin and Peter, 1997]. The sequence variations seen in the analysis of the individual cDNA clones may, however, be a result of tetraploidisation as they would be in the expected homology range. Alternatively, these changes may have either been a result of gene polymorphisms, as RNA was extracted from several goldfish, or as a result of mutations that arose during the PCR reaction. The latter is, however, unlikely as high fidelity polymerases were used to amplify these receptor subtypes [Barnes, 1994].

Like all the cloned GnRH receptors [for review, see Sealfon *et al.*, 1997;], the goldfish receptors are typical G-protein coupled receptors with seven hydrophobic transmembrane domains, as revealed by the amino acid sequence alignment, as well as hydrophobicity plots of the amino acids (figs. 4.2 and 4.3). The goldfish GnRH receptors share a number of features with the cloned mammalian GnRH receptors.

Like the *X. laevis* GnRH receptor (chapter 2) and the catfish GnRH receptor [Tensen *et al.*, 1997] the goldfish GnRH receptors have a low homology to the mammalian receptors in the amino-terminal region. Interestingly the goldfish receptors, as well as the catfish GnRH receptor, have a glycosylation consensus site, which aligns to the human GnRH receptor glycosylation site (Asn^{18}) (fig. 4.2). The GfB receptor subtype as well as the catfish GnRH receptor, however, have two additional glycosylation consensus sites, one at Asn^{11} and one at Asn^{18}. Receptor glycosylation has been shown to be important in receptor expression or stability [Davidson *et al.*, 1995]. It is likely, therefore, that the asparagines at position 23 in the GfB and catfish receptors and position 12 in the GfA receptor are glycosylated as these align to the known glycosylation site of the human GnRH receptor (Asn^{18}). The other two sites in the GfB and catfish GnRH receptors may, however, not necessarily be glycosylated, but it is interesting to note their conservation between the two species. There are three glycosylation consensus sites in the rodent GnRH receptors, but only the two in the amino terminus are glycosylated [Davidson *et al.*, 1995; Arora *et al.*, 1997].

The two cysteines which have been shown to form a disulphide bridge between the amino terminus and EC II in the human GnRH receptor (Cys^{14} and Cys^{200}) [Davidson *et al.*, 1997] are not conserved in the goldfish GnRH receptors. The two cysteines which have been shown to form a disulphide bridge between EC I and EC II, which is conserved in all GPCRs are, however, both conserved in the goldfish GnRH receptors.

The transmembrane domains of both the goldfish GnRH receptor subtypes show a high conservation, both to each other and the mammalian GnRH receptors, showing the importance of these regions to the conformation of the receptor. Like the *X. laevis* pituitary GnRH receptor (chapter 2) and the other cloned non-mammalian GnRH receptors [Tensen *et al.*, 1997; Sun *et al.*, in preparation], the goldfish GnRH receptors have an aspartic acid in the corresponding position of TM II to the asparagine (Asn^{87}) in the human GnRH receptor (fig. 4.2). The asparagine has been shown to interact with an aspartic acid residue in TM VII (Asp^{318}) [Zhou *et al.*, 1993]. Blomenröhr and co-workers [1997] have shown that the aspartic acid in TM II (Asp^{90}) is important for the functioning of the catfish GnRH receptor. The presence of aspartic acid residues in both TMs II and VII in the human GnRH receptor have been shown by mutagenesis to affect the conformation of the receptor, such that it is inactive [Zhou *et al.*, 1994]. The goldfish, and the other non-mammalian GnRH receptors, which have aspartic acids in both TMs II and VII must, therefore, be accommodated by other co-ordinated changes in the transmembrane helices. This will be discussed in greater detail in chapter 5.

All the intracellular loops are highly conserved between the two goldfish receptor subtypes, as well as the catfish GnRH receptor (fig. 4.2), but have a much lower homology with the human GnRH receptor. The IL I of the teleost receptors are is four amino acids shorter than that of the human GnRH receptor. The only residue in IL 1, which is conserved between the human GnRH receptor and the fish receptors, is a serine. This residue could be phosphorylated in all the receptors, as there is a phosphorylation consensus site in this area (basic/basic/X/X/serine). This serine may, thus, be important in receptor desensitisation or internalisation of both the mammalian and non-mammalian receptors. IL III shows a higher conservation to the mammalian GnRH receptors, particularly at the carboxy-terminal end. This region is thought to be important in coupling to G-proteins. The GfA receptor is three amino acids shorter than the GfB receptor in this region.

Extracellular loop I is the most highly conserved of the extracellular loops between the teleost receptors as well as the human GnRH receptor (fig. 4.2). EC II of the teleost receptors is much shorter than that of the mammalian receptors. EC III has a slightly lower homology, but the acidic amino acid residue which was shown to be important in binding to the Arg^{8} of mGnRH, is conserved in the teleost receptors. Like the *X. laevis* pituitary GnRH receptor, the position of the adjacent proline is different and this might contribute to the decreased specificity for mGnRH (chapter 2). The GfA receptor has an acidic amino acid residue at position 285 in EC III (Glu^{285}), the catfish GnRH receptor also has an acidic in this position (Gln^{298}), while the GfB receptor has a basic amino acid in this position (Arg^{298}). Three amino acids 3' to this residue, the situation is reversed, where the GfB receptor has an acidic amino acid (Gln^{301}), while the catfish and GfA receptors have the basic amino acids histidine and lysine respectively. These sequence differences may thus be cancelled out, and thus not have any effect on the receptor, or alternatively they may contribute to differences in the ligand selectivity of the GfA and GfB receptor subtypes. If these differences are important it would be interesting to determine the selectivity of the catfish GnRH receptor to different GnRH analogues particularly with variations in position 8, such as [His^{8}]GnRH and [Tyr^{8}]GnRH.

Both the goldfish receptor subtypes, like all the non-mammalian GnRH receptors cloned to date [chapter 2, Tensen *et al.*, 1997; Sun *et al.*, in preparation] have an intracellular carboxy-terminal tail. The carboxy-terminal tails of GPCRs have been shown to be important in homologous desensitisation [for review, see Benovic *et al.*, 1988]. The carboxy terminal tail of the GfA receptor subtype is slightly shorter than that of the GfB receptor subtype, but both have several phosphorylation consensus sites. The carboxy terminal tails are only 37% conserved between the two goldfish receptor subtypes, which is very low in comparison to the overall amino acid identity of 71%. The tail of the GfB receptor subtype does, however still have a high homology with that of the catfish GnRH receptor subtype (68%).

The effect of different GnRH analogues on the two goldfish receptors transiently expressed in COS-1 cells was investigated by measuring the inositol phosphate production after stimulation. Both receptors coupled to phospholipase C, as both showed a dose dependent increase in inositol phosphate production after GnRH stimulation. The goldfish receptors seemed to show a similar selectivity for the naturally occurring GnRH agonists tested, as cGnRH II was the most potent in stimulating inositol phosphate production, followed by sGnRH and mGnRH. All the GnRHs tested had a lower potency in the GfB receptor compared to the GfA receptor. This may reflect a decreased binding affinity for the GnRH analogues in the GfB receptor, or may be as a result of a lower expression of this receptor in the COS-1 cells.

Several different analogues of GnRH were investigated in order assess if the goldfish receptor subtypes correspond to previous studies on the pharmacology of the receptors on somatotropes and gonadotropes in the goldfish pituitary. The different selectivity for GnRH agonists with variations in position 8 was the most marked. The GfB showed a large decrease in potency for [His^{8}]GnRH, and [Tyr^{8}]GnRH when compared to mGnRH ([Arg^{8}]GnRH), which had a proportionally similar potency for the GfA receptor (table 4.2). This decreased selectivity of GfB for [His^{8}]GnRH and [Tyr^{8}]GnRH is similar to that seen in the GtH releasing activity of the gonadotropes [Habibi *et al.*, 1992].

Studies on the inhibition of both sGnRH and cGnRH II stimulated inositol phosphate production with the analogues E, C and L, did not correlate with previous studies [Murthy and Peter, 1994; Murthy *et al.*, 1994; 1993]. These studies showed that analogue E ([Ac-Pro^{1},D-Fpa^{2},D-Trp^{3,6}]GnRH) is a pure antagonist for both salmon and cGnRH II stimulated GtH II and GH release, while analogue C ([Ac-Pro^{1}, D-Fpa^{2}, D-Trp^{3,6},Trp^{7},Leu^{8}]GnRH) inhibits salmon and cGnRH II stimulated GtH release, but stimulates GH release and analogue L ([Ac-D-Nal^{1}, D-Cpa^{2}, D-Pal^{3},Arg^{5},D-Pal^{6},D-Ala^{10}]GnRH) weakly stimulates GtH release but inhibits GH release. Based on the selectivity for GnRHs with variations in position 8, GfB may be the GtH-releasing receptor, while the GfA receptor

subtype, may be the GH-releasing receptor. One would, therefore, expect analogue E to be a true antagonist for both the A and B receptor subtypes, analogue C to be an antagonist for the GfB receptor and an agonist for the GfA receptor, and analogue L to be an antagonist for the GfA receptor and an agonist for the GfB receptor. The three analogues were, however, pure antagonists for both the receptor subtypes as they all inhibited both cGnRH II (data not shown) and sGnRH stimulated inositol phosphate production (fig. 4.5). Despite the difference in selectivity of [His^8]GnRH and [Tyr^8]GnRH corresponding to the studies on GtH II and GH release, these antagonists do not appear to distinguish the two receptor subtypes.

The two goldfish receptor subtypes also have a different selectivity for the [D-Arg^6]GnRH analogues. The GfA receptor, unlike the GfB receptor, seems to have no increased potency for the cGnRH II-like analogue [D-Arg^6,Trp^7,Tyr^8]GnRH, while the opposite is true for the sGnRH-like analogue, [D-Arg^6,Trp^7,Leu^8]. These analogues, thus, distinguish between the GfA and GfB subtypes, but there is no published data on the effect of these analogues on GtH II and GH release.

In situ hybridisation studies on goldfish brain, ovaries and liver have been conducted by Dr R.E. Peter (University of Alberta, Edmonton, Canada), using anti-sense probes designed to the A and B receptor subtypes [Illing *et al.*, in preparation]. These studies have shown that both the GfA and GfB receptor subtypes are both expressed in the pituitary, with overlapping expression in gonadotropes and a limited overlap in somatotropes. There were no significant differences between the expression of the two receptors in the pituitary. Both receptors showed extensive expression in the brain but the GfA receptor showed unique additional hybridisation to several brain areas, including the nucleus anterior *tuberis*, the optic tectum and the *area ventralis telecephali,* as well as interstitial and theca cells of the ovary and hepatocytes in the liver.

In conclusion therefore, the two receptors, which have been cloned and characterised, are both expressed in the goldfish brain and pituitary. Although

there is some evidence, based on the amino acid sequence homology with the GtH-releasing receptor in catfish and pharmacological data with position 8 substituted analogues, that these receptors represent the two receptors for GH- and GtH- release in the goldfish. These results were, however, not confirmed with the pharmacological studies using analogues E, C and L, which have previously been shown to distinguish the two receptors [Murthy and Peter, 1994; Murthy *et al.*, 1994; 1993]. The two receptors may, however, correspond to the GH and GtH releasing receptors and that the differences in the *in vitro* bioassays and the inositol phosphate studies in transfected COS-1 cells may be due to a complex interplay between agonist, receptor, G-proteins and second messenger systems in the goldfish pituitary gonadotropes and somatotropes which cannot be mimicked in the transfected system. *In situ* hybridisations with probes designed to the two receptor subtypes, however, did show the localisation of both the receptors to gonadotropes but not significantly to somatotropes, but rather showed a distinct distribution in the brain, ovaries and liver. Thus either a third GnRH receptor with GH-releasing activity may exist in the goldfish pituitary, or alternatively GnRH has a paracrine effect in stimulating GH-release. The two receptor subtypes described here, may play an important role in the regulation of pituitary hormones, and may have an additional role, possibly in reproductive behaviour.

Chapter 5

Concluding Discussion

The field of comparative endocrinology has provided useful insights in the study of both non-mammalian and mammalian systems, at several levels. The present study on gonadotropin-releasing hormone receptors, has made use of two model systems, which are comparable to the mammalian system, but have unique characteristics which should provide useful insights into the structure and function of GnRH receptors.

Structural determinants of agonist and antagonist binding are vital for the design of therapeutic agents. A detailed analysis of the cloned mammalian GnRH receptors has been useful in determining amino acid residues important for receptor conformation, ligand binding and specificity, and motifs or residues required for G-protein coupling [for review, see Sealfon *et al.*, 1997]. These studies were done using receptor site-directed mutagenesis whereby the effect of changing key amino acids which were identified by either alignments with other related G-protein coupled receptors, or intuitive insights based on receptor/ligand specificity. An alternative approach to identify important amino acid residues is to observe co-ordinated structural/functional changes in evolutionarily distinct GnRH receptors. Amino acid residues or motifs, which are conserved, may either be important for the overall conformation of the receptor, ligand binding or G-protein interactions. Residues, which are not conserved may be unimportant, may contribute to unique receptor features, such as ligand specificity or may represent co-ordinated changes at several sites. Residues, which are conserved amongst the non-mammalian GnRH receptors but not the mammalian receptors may also provide useful insights into the structural/functional relations of GnRH receptors. A comparison of the primary sequence of the GnRH receptors from *X. laevis*, goldfish (this thesis) and catfish [Tensen *et al.*, 1997] to the mammalian GnRH receptors has provided many insights into the structure and function of GnRH receptors.

There are several interesting features, which are revealed by the conservations between the non-mammalian GnRH receptors described in this work, the catfish

GnRH receptor [Tensen *et al.*, 1997] and the mammalian GnRH receptors (fig. 5.1). In this discussion the numbering system is based on the human GnRH receptor.

The asparagine (Asn^{18}) in the amino-terminus, which has been shown to be glycosylated in the mammalian receptors [Davidson *et al.*, 1995; 1996(a); Arora *et al.*, 1997], is conserved. Glycosylation of the receptor has been shown to improve expression, but does not seem to affect ligand binding or signal transduction [Davidson *et al.*, 1995; Arora *et al.*, 1997]. The glycosylation of the non-mammalian receptors may, therefore, also be important for optimal receptor expression.

The transmembrane domains (TM) show the highest conservation, with TM VI being the most conserved. This high conservation is expected as the transmembrane domains amongst different GPCRs usually show a high level of conservation [Probst *et al.*, 1992; for review, see Strader *et al.*, 1994]. Amongst the conserved residues in the transmembrane domains are the highly conserved proline residues in TM II, TM IV, TM V, TM VI and TM VII [Baldwin, 1993]. Other highly conserved residues among GPCRs, which are conserved between the non-mammalian and mammalian GnRH receptors include the asparagine (Asp^{53}) in TM I, the tryptophan (Trp^{164}) and serine (Ser^{167}) in TM IV and the tyrosine (Tyr^{323}) in TM VII. Asp^{138} and Arg^{139} at the border of TM III and IL II which are important for agonist induced activation [Ballesteros *et al.*, 1998] and internalisation [Davidson *et al.*, 1994(a); Arora *et al.*, 1997] are also conserved. The two isoleucines located one turn up or down in the α-helix from Arg^{139}, (Ile^{135} and Ile^{143}) are thought to 'cage' the arginine limiting the movement of the receptor in adopting the active or inactive conformations [Ballesteros *et al.*, 1998], are also conserved in the non-mammalian GnRH receptors. Ile^{143} is conserved, while Ile^{135} is conserved in the *X. laevis* pituitary and goldfish A GnRH receptors and a valine in the catfish and goldfish B GnRH receptors. Valine is also a β-branched amino acid, and this is thus a conservative change.

In all the GnRH receptors the residues shown to be important in ligand binding, are conserved. These residues include Asn^{102} at the top of TM II, which has been shown to interact with the glycine amide of GnRH [Davidson *et al.*, 1996(b)] and Asp^{98} in TM II and Lys^{121} in TM III, which have been shown to interact with the histidine in position two of GnRH [Rodic *et al.*, 1996; Zhou *et al.*, 1995].

The intracellular loops (IL) are poorly conserved. IL I of all the receptors, however, consists of predominantly basic amino acid residues. The conservation of Ser^{74}, is interesting as this is a consensus phosphorylation site for protein kinase C in all the receptors (basic/X/X/Ser/X/X/basic). The relatively high conservation of the carboxy-terminal portion of IL III, may be as a result of the importance of this region in G-protein coupling. Ala^{261}, which has been directly implicated in G-protein coupling, is also conserved [Myburgh *et al.*, 1998].

Of the extracellular loops, EL I is almost 100% conserved, while the central portion of EL II is highly conserved the amino- and carboxy-termini of this loop are poorly conserved, and EL III shows almost no conservation. Several of the residues of EL I such as Trp^{107}, Gly^{110}, Leu^{112}, Cys^{114} are conserved amongst many GPCRs [Probst *et al.*, 1992]. The high conservation in this loop may, therefore, be as a result of its importance to the general conformation of the receptors. The cysteines, which form the highly conserved disulphide bridge between Cys^{114} of EL I and Cys^{196} of EL II [for review, see Strader *et al.*, 1994] are also conserved.

There are also several features of the non-mammalian GnRH receptors, which are distinct from the mammalian receptors (fig. 5.2). The most noticeable of these is the presence of an intracellular carboxy-terminal tail in all the non-mammalian receptors. Studies on the chicken [Sun *et al.*, in preparation] and catfish [Tensen *et al.*, 1997] GnRH receptor carboxy-terminal tails have shown them to be important for ligand induced rapid desensitisation [Heding *et al.*, 1998] and internalisation [Heding *et al.*, 1998; Pawson *et al.*, 1998]. Addition of the catfish GnRH receptor carboxy-terminal tail to the rat GnRH-receptor increased expression of the rat receptor [Lin *et al.*, 1998]. Truncation of the catfish GnRH receptor tail, however, reduced agonist binding [Blomenröhr *et al.*, 1997]. The carboxy-terminal tails of the non-mammalian GnRH receptors may, thus, be important for rapid ligand-induced receptor desensitisation and internalisation and may have direct or indirect affects on receptor expression and ligand binding. The mammalian GnRH receptors, which lack a carboxy-terminal tail do not show a rapid desensitisation after agonist stimulation like other GPCRs [Davidson *et al.*, 1994], but do show a long-term agonist induced down regulation [for review, see Millar *et al.*, 1987]. The lack of rapid desensitisation due to

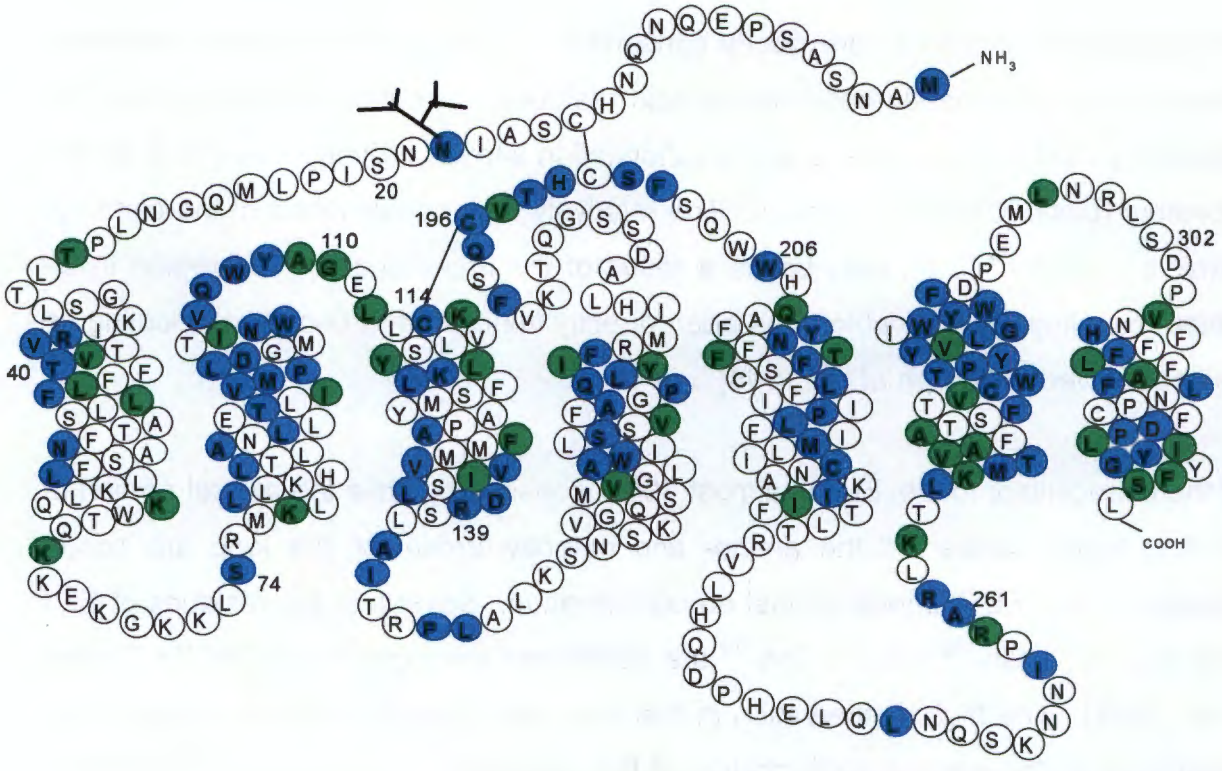


Figure 5.1. Diagram of the human GnRH receptor, showing amino acid residues, which are conserved with the *X. laevis* pituitary GnRH receptor (chapter 2), the partial clone of the second *X. laevis* GnRH receptor (chapter 3), the goldfish A and B GnRH receptor subtypes and the catfish GnRH receptor [Tensen *et al.*, 1997] (blue) and conservative changes (green).

the absence of a carboxy-terminal tail [Heding *et al.*, 1998; Lin *et al.*, 1998; Pawson *et al.*, 1998] to prevent receptor desensitisation and down regulation may be important in allowing the protracted LH-surge required for ovulation. This lack of receptor desensitisation is, however, not responsible for the LH surge, which is initiated by an increased secretion of estradiol-17β, and appears to be due to a complex interplay of increased GnRH secretion coupled to an increased pituitary response, possibly by an increased expression of the GnRH receptor. Estrogen has previously been shown to increase GnRH receptor mRNA levels [Sealfon *et al.*, 1990; Quinones-Jenab *et al.*, 1996]. The carboxy-terminal tails have several phosphorylation consensus sites, which may be involved in receptor desensitisation via phosphorylation by serine/threonine kinases. The carboxy-terminal tails may also be important in the stabilisation of receptor structure by providing an additional

scaffold possibly by the palmitoylation of Cys residues or the insertion of hydrophobic residues of the tail into the membrane [for review, see Strader *et al.*, 1994]. It is interesting to note that all the non-mammalian GnRH receptors lack the second disulphide bridge thought to occur in the human GnRH receptor between Cys residues in the NH_2-terminus and EL III [Davidson *et al.*, 1997]. This second disulphide bridge may have, therefore, evolved to compensate for the lack of carboxy-terminal tail. The mammalian GnRH receptors are unique in that the IL I is much longer than that of the non-mammalian GnRH receptors and other GPCRs [Probst *et al.*, 1992; Tsutsumi *et al.*, 1992]. This may also have evolved in the mammalian GnRH receptors to accommodate the lack of carboxy-terminal tail. Related to this is the lack of conservation of the second protein kinase C phosphorylation consensus site in IL I of the mammalian receptors at Thr^{64}, with any of the non-mammalian receptors.

A second different feature is the presence of an Asp residue in TM II of all the non-mammalian receptors in accordance with most GPCRs and, which is an Asn in the mammalian GnRH receptors (Asn^{87}). The mammalian GnRH receptors appear to have undergone a reciprocal change between Asn^{87} and Asp^{318} in TM VII when compared to other GPCRs. This exchange suggests an interaction of the side chains of these residues in TM II and TM VII [Zhou *et al.*, 1994]. Mutagenesis of Asn^{87} to an Asp requires the simultaneous mutation of Asp^{318} to and Asn to maintain ligand binding [Zhou *et al.*, 1994]. It is, thus, interesting to note that the non-mammalian receptors have Asp residues on both the corresponding positions of TMs II and VII. Mutations of the Asp in TM VII of the catfish GnRH receptor to an Asn could still bind GnRH [Blomenröhr *et al.*, 1997]. Mutations of the Asp in TM II (Asp^{90}) to an Asn, however, showed almost no binding [Blomenröhr *et al.*, 1997]. The Asp in position 90 of TM II in the catfish GnRH receptor, thus, seems to be critical for receptor functioning, and this is likely to be true for all the non-mammalian GnRH receptors. It is possible that there are other co-ordinated changes in the non-mammalian receptors, which support the Asp residues in both TMs II and VII. Most noticeable differences between the non-mammalian and mammalian GnRH receptors is that the mammalian receptors have a basic Lys at position 81, which is approximately two turns above the Asn^{87}, while the non-mammalian receptors all have hydrophobic residues in the corresponding position (Ala in the catfish; Gly in GfB; Ile in GfA and

Leu in *Xl*.A). Adjacent to the Lys residue is a His. The non-mammalian receptors all have a Ser in the corresponding position. Also one turn up in the α-helix from Asn^{87}, the mammalian receptors have an acidic glutamic acid (Glu^{90}), while the non-mammalian GnRH receptors have either a Met (teleost GnRH-Rs) or a Val (*X. laevis* pituitary GnRH-R), which are both hydrophobic non-polar residues. The presence of these three extra hydrophobic amino acids in TM II of the non-mammalian GnRH receptors may act individually or in concert, to alter the conformation of the α-helix in such away that it is able to support Asp interactions between TMs II and VII.

The acidic residue in EL III, Glu^{301} of the mouse GnRH receptor (or Asp^{302} of the human GnRH receptor), is crucial for selectivity of Arg^{8} in mammalian GnRH [Flanagan *et al.*, 1994]. It was initially thought that the non-mammalian receptors which were shown not to have a high specificity for mGnRH [for review, see King and Millar, 1997], would not have an acidic residue in the corresponding position to the Glu^{301} of the mouse GnRH receptor. Surprisingly an acidic residue is consistently conserved in this position of the non-mammalian GnRH receptors, except in the *X. laevis* B receptor subtype. An in depth pharmacological characterisation of the amphibian and teleost GnRH receptors, however, revealed that contrary to previous studies [for review, see King and Millar 1997] the receptors do have a limited selectivity for Arg^{8}. This is revealed by the increased potency of the non-mammalian receptors for mGnRH, in comparison to that of cGnRH I ([Gln^{8}]GnRH). This selectivity for mGnRH in the non-mammalian GnRH receptors is, however, reduced in comparison to that of the mammalian GnRH receptors, therefore suggesting other changes, possibly affecting the interaction between the basic Arg in position 8 and the acidic residue in EL III of the receptor. Interestingly a proline residue precedes the acidic residue in the non-mammalian GnRH receptors, while a proline follows the acidic residue in the mammalian GnRH receptors. Because prolines are known to affect the secondary structure of proteins by inducing β-turns or disrupting α-helices [Chou and Fasman, 1978], this difference may drastically alter the conformation of EL III and effect the orientation of the side chain of the acidic residue, and hence ligand selectivity. The *X. laevis* B GnRH receptor subtype, which has two adjacent proline residues in the corresponding position, and lacks the acidic residues (fig. 5.2), may therefore, have a drastically different ligand selectivity to both the other non-

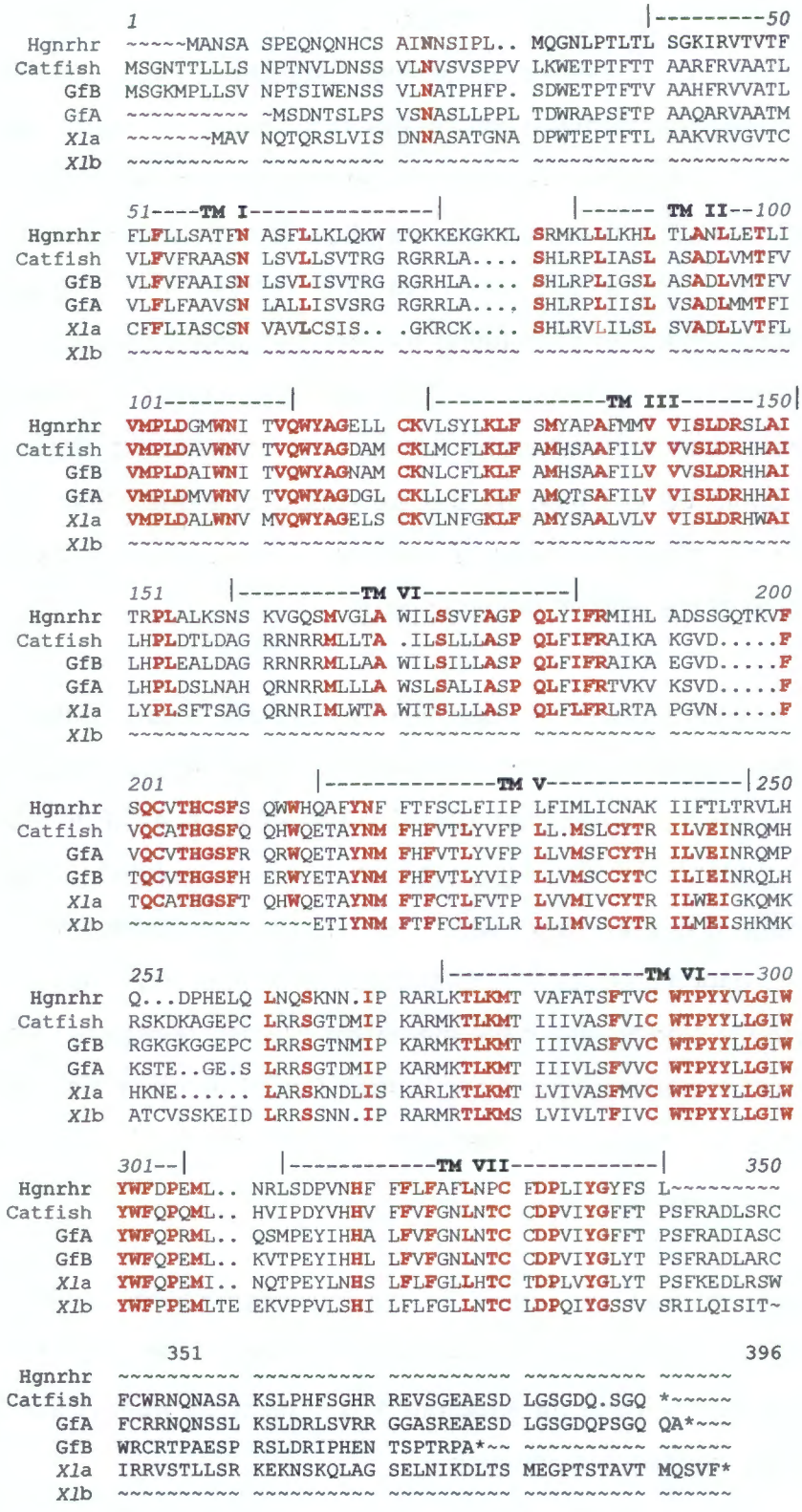


Figure 5.2. Alignment of the amino acid sequences of the human (hgnrhr), catfish, goldfish A and B (GfA and GfB), *X. laevis* A and B (Xl A and Xl B) GnRH receptors. Amino acid residues shown in red are conserved between all the GnRH receptors shown here.

mammalian and the mammalian GnRH receptors. The effect of the positioning of the acidic amino acid in EL III relative to proline, may be reflected by the reduced selectivity of mGnRH, and furthermore in the differences in ligand selectivity of cGnRH II versus mGnRH in the non-mammalian and mammalian GnRH receptors. The non-mammalian GnRH receptors do not show a high selectivity for mGnRH, however, they all seem to be a highly selective for cGnRH II. This is reflected by the high potency of this peptide in stimulating inositol phosphate production in COS-1 cells transiently expressing the receptors. This possibility can be explored further by the use of chimeric receptors, whereby the agonist selectivity of the mammalian GnRH receptors could be changed by the exchange of extracellular loops of the mammalian GnRH receptors with those of non-mammalian receptors, thereby adding to our understanding of the agonist binding pocket.

In addition to showing differences in selectivity for naturally occurring forms of GnRH the non-mammalian GnRH receptors tested, all show partial agonism to the mammalian GnRH receptor antagonist 135-18 ([Ac-*D*-Nal^{1}, *D*-4-ClPhe^{2}, *D*-Pal^{3}, Ile^{5}, *D*-IsopropylLys^{6,8}, *D*-Ala^{10} NH_2]GnRH) (data not shown for goldfish GnRH receptor subtypes). Although it is not known which residues in the non-mammalian GnRH receptors may be responsible for this interaction this difference may be useful in defining the antagonist binding site in the mammalian GnRH receptors. The chimeric exchange of the extracellular loops of the human GnRH receptor with the *X. laevis* pituitary GnRH receptor, have implicated extracellular loop II [Ott TR, unpublished results].

A detailed analysis of the sequences of the non-mammalian GnRH receptors, coupled to pharmacological characterisations, therefore, lays the foundation for future studies involving the receptor structure and stability as well as the delineation of agonist and antagonist binding domains.

The isolation of multiple GnRH subtypes in vertebrates and functional studies of these peptides have implicated them in several regulatory roles in addition to the regulation of the hypothalamic/pituitary gonadal axis [for review, see King and Millar, 1997]. The function of the highly conserved cGnRH II, which has only recently been described in primates and humans [Lescheid *et al.*, 1997; White *et al.*, 1998] is

unclear, but its high conservation throughout evolution suggests a stringent selection, possibly due to multiple functions, and possibly constrained by the interaction with different receptor subtypes. The occurrence of one or more variants of GnRH, as well as the highly conserved cGnRH II lays the foundation for the study of multiple GnRH receptor subtypes.

In amphibia, cGnRH II has been proposed to be involved in neuromodulation or neurotransmission [King *et al.*, 1994a] and has been implicated in the inhibition of M-current [Jan and Jan, 1980; 1982; Jones 1987]. M-current inhibition has been shown to have an effect on neuron excitability [Kirkwood and Lissman, 1992]. The occurrence of cGnRH II and cGnRH II specific binding sites in extracts of sympathetic ganglia, described in this thesis, provides further evidence that cGnRH II may be the endogenous neurotransmitter in this response. Thus, in the amphibian brain, one would predict the occurrence of two GnRH receptor subtypes. One, which is involved in the release of LH and FSH from the pituitary, while the other may inhibit M-current in the sympathetic ganglia. Two GnRH receptor subtypes were isolated from *X. laevis* genomic DNA. The full-length cDNA for the one was cloned from the pituitary, while a processed, but incomplete transcript was cloned from sympathetic ganglia cDNA. Although no full-length transcripts were identified, the fact that a processed transcript is present in cDNA from the sympathetic ganglia implies an expression of this receptor in these neurons, which is substantiated by the detection of specific cGnRH II binding sites. The GnRH dependent M-current inhibition has been described in rats [Owen *et al.*, 1990] and may, therefore, be a widespread mechanism for controlling neuron excitability. Possible homologues to this receptor have been detected in the lizard (*Agama atra*) by PCR on genomic DNA (Dr E. Rumbak) and in the human by database interrogation (Prof. R. Millar). Due to the high conservation of these putative GnRH receptor subtypes in amphibians, reptiles and mammals, it is unlikely that they are unexpressed pseudogenes.

The goldfish is also a unique model system in that two distinct GnRH receptor subtypes may facilitate the release of GH and GtH from somatotropes and gonadotropes respectively. Two GnRH receptor subtypes were identified, which have different primary structures and co-ordinated with these differences, distinct pharmacologies. There is no clear correlation between the pharmacology of these

cloned receptor subtypes and the GtH and GH-releasing activity of GnRH analogues, but expression patterns as determined by *in situ* hybridisations (Dr R.E. Peter, University of Alberta, Edmonton), imply a role in pituitary hormone secretion and possibly reproductive behaviour.

Although the occurrence of multiple GnRHs in a single species suggests the co-ordinated occurrence of multiple receptor subtypes, this is the first description of multiple GnRH receptor subtypes in a single species. Although only one full-length receptor was isolated from *X. laevis*, there is convincing evidence for the presence of a second receptor, although its expression remains to be elucidated. In the goldfish, however, two receptors were cloned, and these have distinct pharmacologies and differential expression in the brain, gonads and liver. The sequence corresponding to EC III of two receptor subtypes have also been cloned from the genomic DNA of the lizard (*Agama atra*) and the zebrafish (*Brachydanio rerio*) by Dr E Rumbak. From the sequence alignment of all the receptor subtypes, there appear to be two receptor groups, designated type I and type II, which may have arisen via the duplication of an ancestral gene (fig. 5.3). The first group, designated type I, includes the mammalian, the chicken [Sun *et al.*, in preparation], the *X. laevis* A, one of the lizard receptor subtypes, the catfish, and the two goldfish and zebrafish GnRH receptors. Despite the fact that no classifications can be made, based on the homology data, these receptors have to be grouped into two groups, type Ia and type Ib, due to the multiple receptor subtypes in the goldfish and zebrafish. The human GnRH receptor appears to be equally unrelated to both the type I and type II receptors (fig. 5.4), but has been grouped with the type I receptors due to its sequence similarities in EL III. The type II receptors include the *X. laevis* B receptor, the second lizard receptor subtype and the second human GnRH receptor (HGnRHRII) [Millar *et al.*, submitted]. These receptors show a high homology to each other and a distinctive sequence in EL III consisting of two adjacent proline residues (fig. 5.2). Although this classification is based on the limited sequence to EL III, as the full-length sequences are not available for all the receptors, it is likely to be representative of the full-length receptors for two reasons. Firstly, because EL III is thought to be an important determinant in ligand selectivity, and secondly because the sequence information in this domain is unbiased by the low conservation in the NH_2- and carboxy-termini domains and the highly conserved transmembrane domains. A similar pattern is

obtained using the full-length sequences of the human, chicken [Sun *et al.*, in preparation], catfish [Tensen *et al.*, 1997], goldfish A and B and *X. laevis* pituitary GnRH receptors (this thesis) as well as the partial clones to the *X. laevis* B (chapter 3, this thesis) and the human type II (HGnRHRII) [Millar *et al.*, submitted] putative GnRH receptor subtypes (fig. 5.4).

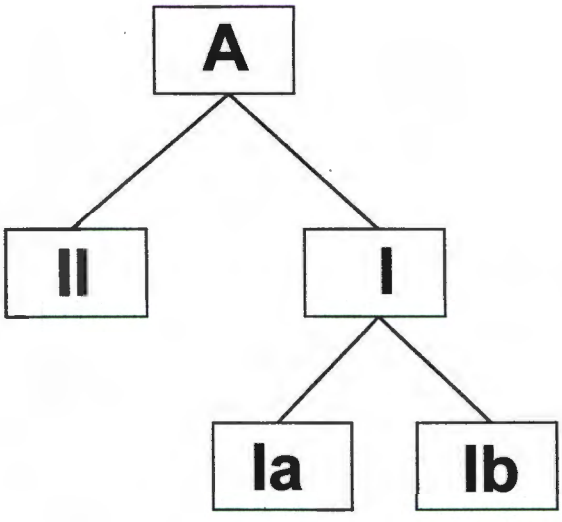


Figure 5.3 A possible evolutionary scheme for the putative GnRH receptor subtypes. A primitive ancestral gene may have undergone a gene duplication forming two receptor groups, designated type I and type II. The type I receptor subtype, may have subsequently undergone a further duplication (at least in some teleosts) forming the type Ia and type Ib receptor subtypes.

Cloning and characterisation of multiple GnRH receptor subtypes from the amphibian, *X. laevis* and the teleost, *C. auratus*, has provided some useful insights into the understanding of both non-mammalian and mammalian GnRH receptors. This work lays the foundation for the identification of receptor subtypes in other vertebrate species, including mammals. The sequence information has already been used to identify a possible homologue, to the type II receptor subtype in *X. laevis,* in the human. Analysis of the primary structure of the non-mammalian GnRH receptors described in this thesis, has revealed several features which are unique from each other and the mammalian GnRH receptors. Coupled to these changes are distinct pharmacologies and ligand selectivities. Characterisation using GnRH analogues has revealed that these receptors, in contrast to previous studies do show a selectivity for naturally occurring GnRHs, in particular chicken GnRH II. This work therefore lays the

groundwork for future studies on the interaction of GnRH and its receptor, by the use of receptor site-directed mutagenesis studies and the analysis of non-mammalian/mammalian chimeric GnRH receptors.

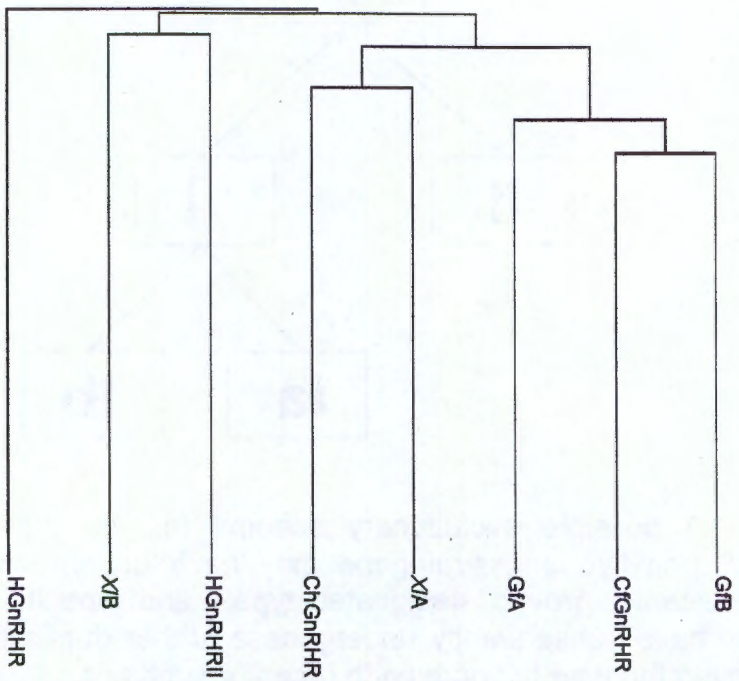


Figure 5.4. Dendogram of the relatedness between the human GnRH receptors, type I (HGnRHR) and type II (HGnRHRII) [Millar *et al.*, submitted], Chicken (ChGnRHR) [Sun *et al.*, in preparation], Catfish (CfGnRHR) [Tensen *et al.*, 1997], Goldfish A and B receptor subtypes (GfA and GfB) and the *X. laevis* A and B receptor subtypes (*Xl*A and *Xl*B). Full-length sequences were used where available, only partial sequences of HGnRHRII and *Xl*B were used. Relatedness determined using the Wisconsin Package, Version 9.0, Genetics Computer Group (GCG), Madison, Wisconsin (Multiple sequence alignment, Pileup).

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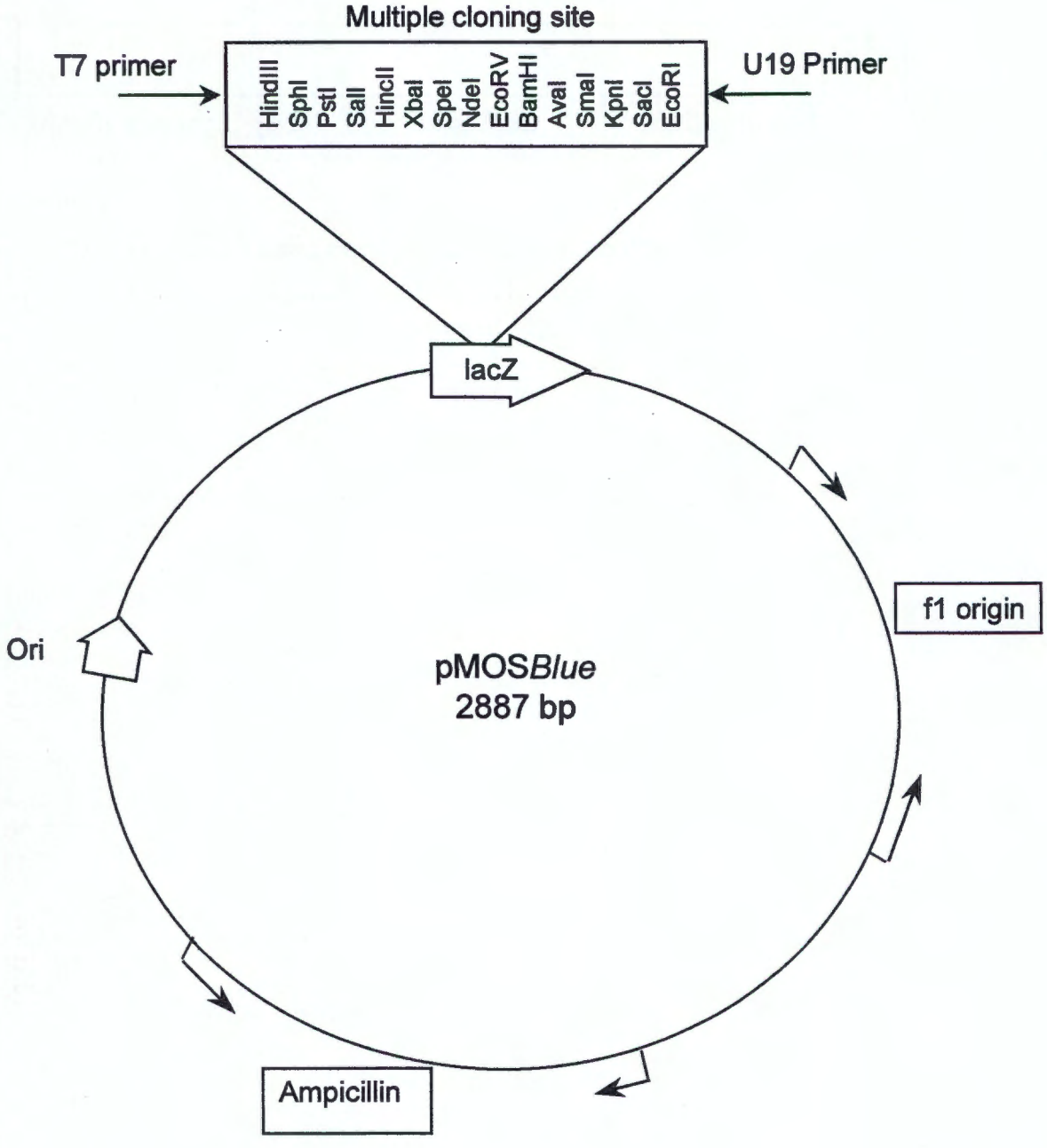
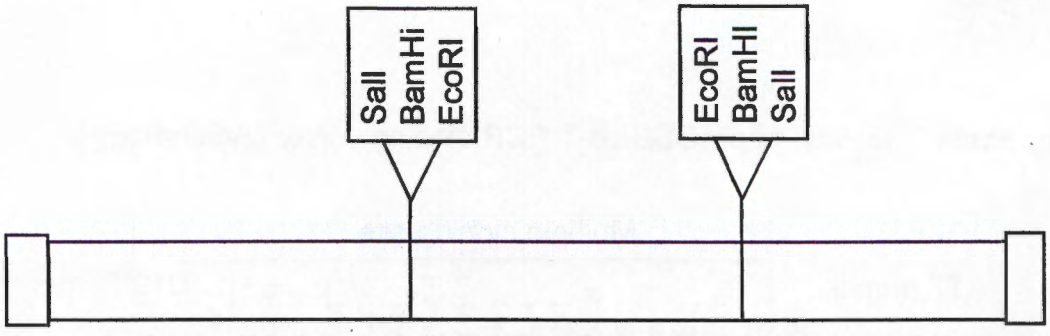
Appendices

Appendix 7.1: Map of pMOS*Blue*-T PCR cloning vector (Amersham)



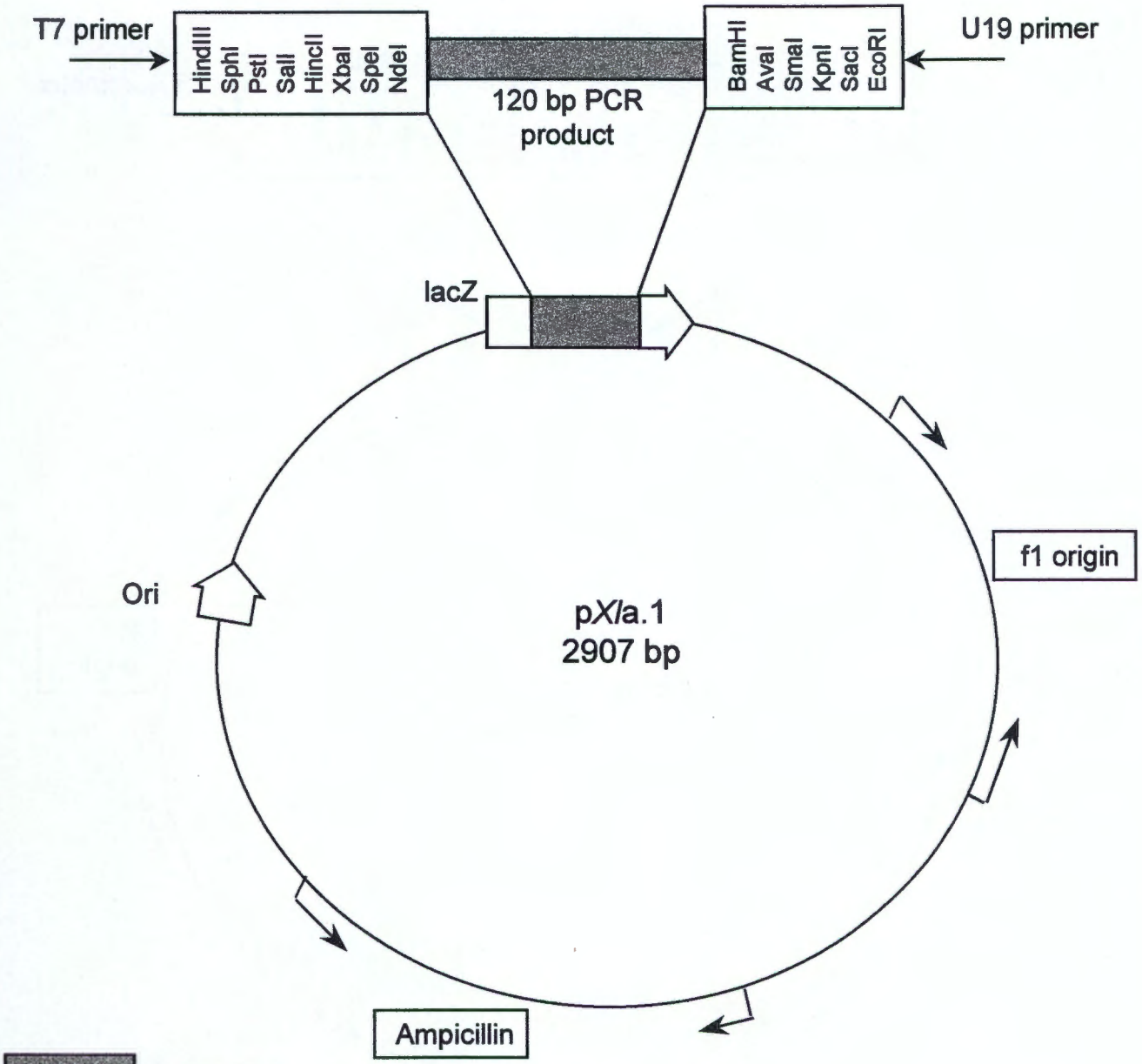
Appendix 7.2: Map of Bacteriophage Lambda EMBL4 vector, showing the mulitple cloning regions



5'.....ggatc tgggt cgacg gatcc gggga attcc cagat cc.....3'

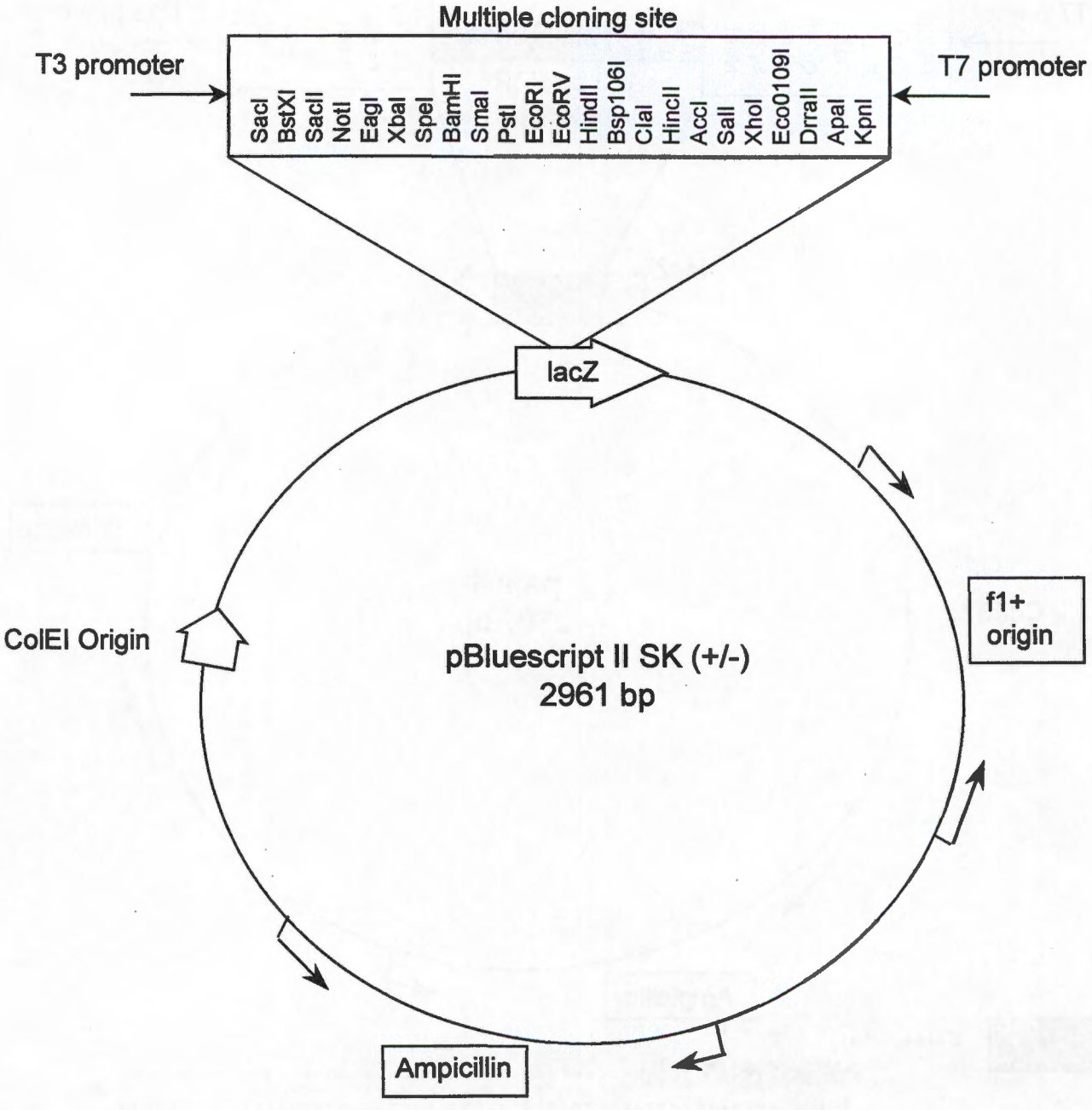
SalI *BamHI* *EcoRI*

Appendix 7.3: Map of p*Xl*a.1 showing the nucleotide and translated amino acid sequences of the insert.

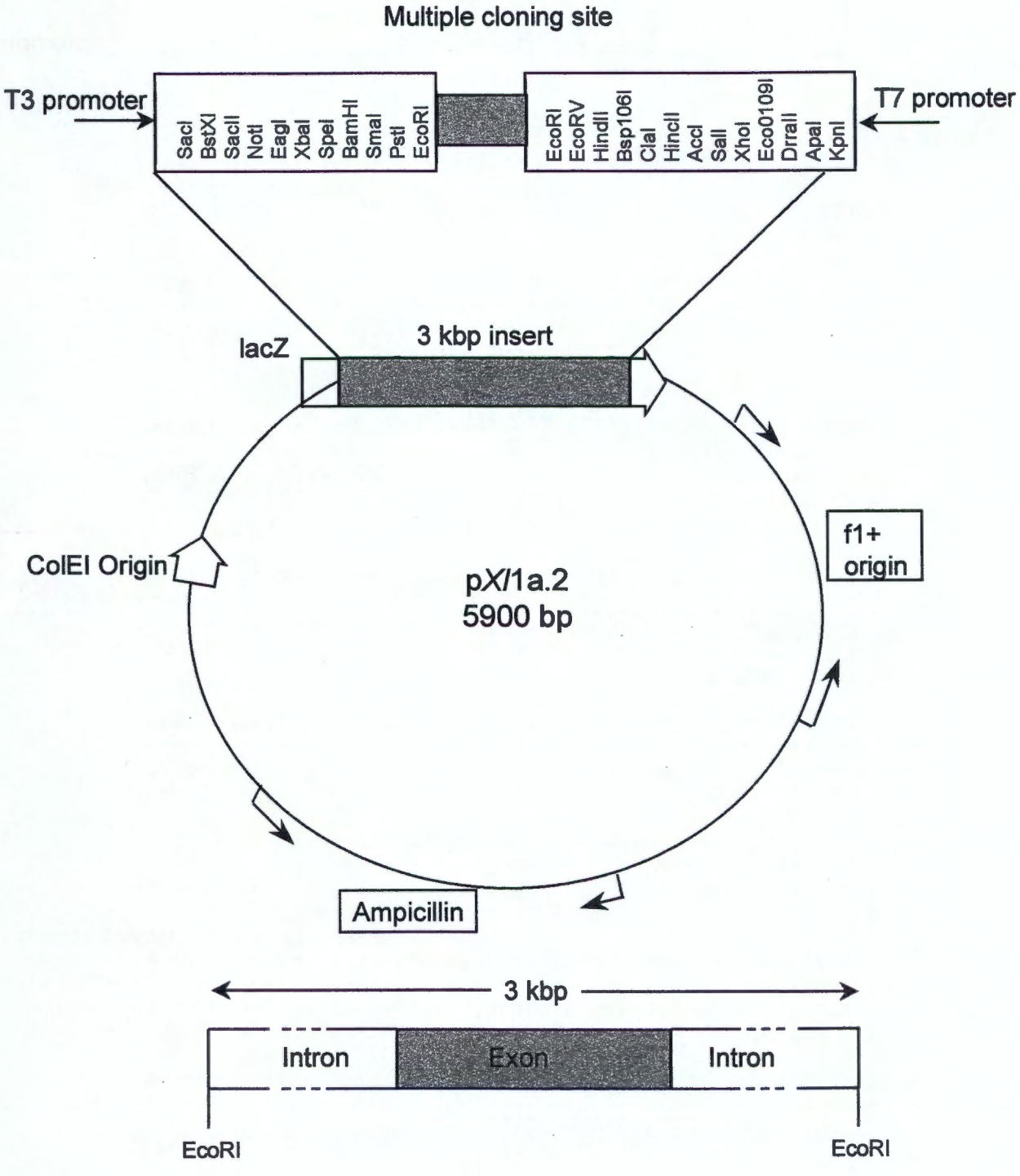



```
                                                                     60
1   ---------+---------+---------+---------+---------+---------+
    cctgataccatgaccaaggtcggactctactagttggtttgaggactcatggaattggtg

    G  I  W  Y  W  F  Q  P  E  M  I  N  Q  T  P  E  Y  L  N  H  -
          JH 5s

    agtcttttcctctttggtctgctgcacacctgcaccgatcccttatctac
                                                                    112
61  ---------+---------+---------+---------+---------+---------+--
    tcagaaaaggagaaaccagacgacgtgtggacgtggctaggggaatagatg

    S  L  F  L  F  G  L  L  H  T  C  T  D  P  L  I  Y  -
                                       JH 6a2
```

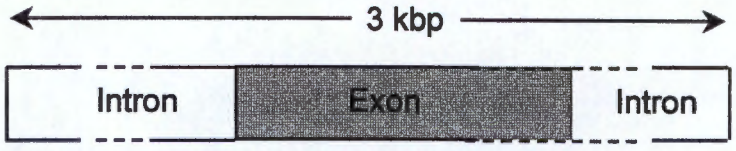
Appendix 7.4: Map of pBluescript II SK(+/-) phagemid, showing the multiple cloning site.



Appendix 7.5a: Map of p*X*l1a.2 containing a 3 kbp genomic DNA fragment cloned into the EcoRI site of pBluescript II SK(+/-).



Appendix 7.5b: Sequence of p*X*l1a.2, showing the primers used for amplification of cDNA



Intron ← Exon

```
    tgtgagcttgcaagaagtaaaaatgatctcatttcaaaggcaagactaaaaaccctaaag
  1 ---------+---------+---------+---------+---------+---------+ 60
    acactcgaacgttcttcatttttactagagtaaagtttccgttctgatttttgggatttc
    C  E  L  A  R  S  K  N  D  L  I  S  K  A  R  L  K  T  L  K  -

   atgaccctagttattgtggcatcctttatggtctgctggacccccctattacctactcgga
 61 ---------+---------+---------+---------+---------+---------+ 120
   tactgggatcaataacaccgtaggaaataccagacgacctgggggataatggatgagcct
   M  T  L  V  I  V  A  S  F  M  V  C  W  T  P  Y  Y  L  L  G  -
```

*X*l1a.4s

```
    ctatggtactggttccagcctgagatgatcaaccaaactcctgagtaccttaaccacagt
121 ---------+---------+---------+---------+---------+---------+ 180
    gataccatgaccaaggtcggactctactagttggtttgaggactcatggaattggtgtca
    L  W  Y  W  F  Q  P  E  M  I  N  Q  T  P  E  Y  L  N  H  S
```

*X*l1a.3a

```
    cttttcctctttggtctgctgcacacctgcaccgatccccttgtctatggactttacact
181 ---------+---------+---------+---------+---------+---------+ 240
    gaaaaggagaaaccagacgacgtgtggacgtggctaggggaacagatacctgaaatgtga
    L  F  L  F  G  L  L  H  T  C  T  D  P  L  V  Y  G  L  Y  T  -
```

*X*l1a.2a

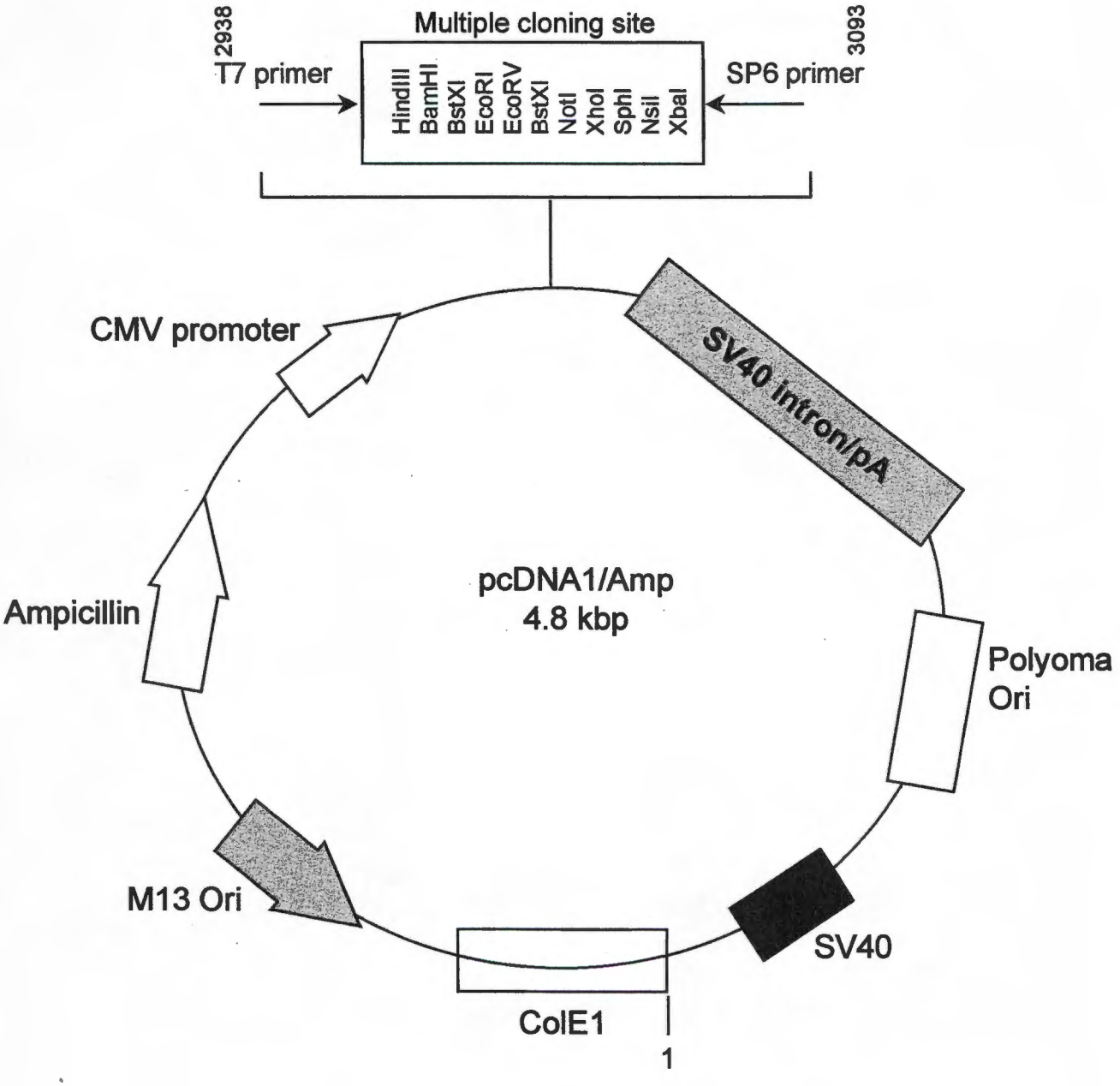
```
    ccctcattcaaagaggacctgcgatcatggatcagaagagtgagcactctactgtctaga
241 ---------+---------+---------+---------+---------+---------+ 300
    gggagtaagtttctcctggacgctagtacctagtcttctcactcgtgagatgacagatct
    P  S  F  K  E  D  L  R  S  W  I  R  R  V  S  T  L  L  S  R  -

    aaagaaaaaaacagtaagcagctagctggctcagagctgaatatcaaagatcttacctca
301 ---------+---------+---------+---------+---------+---------+ 360
    tttctttttttgtcattcgtcgatcgaccgagtctcgacttatagtttctagaatggagt
    K  E  K  N  S  K  Q  L  A  G  S  E  L  N  I  K  D  L  T  S  -
```

3' untranslated region

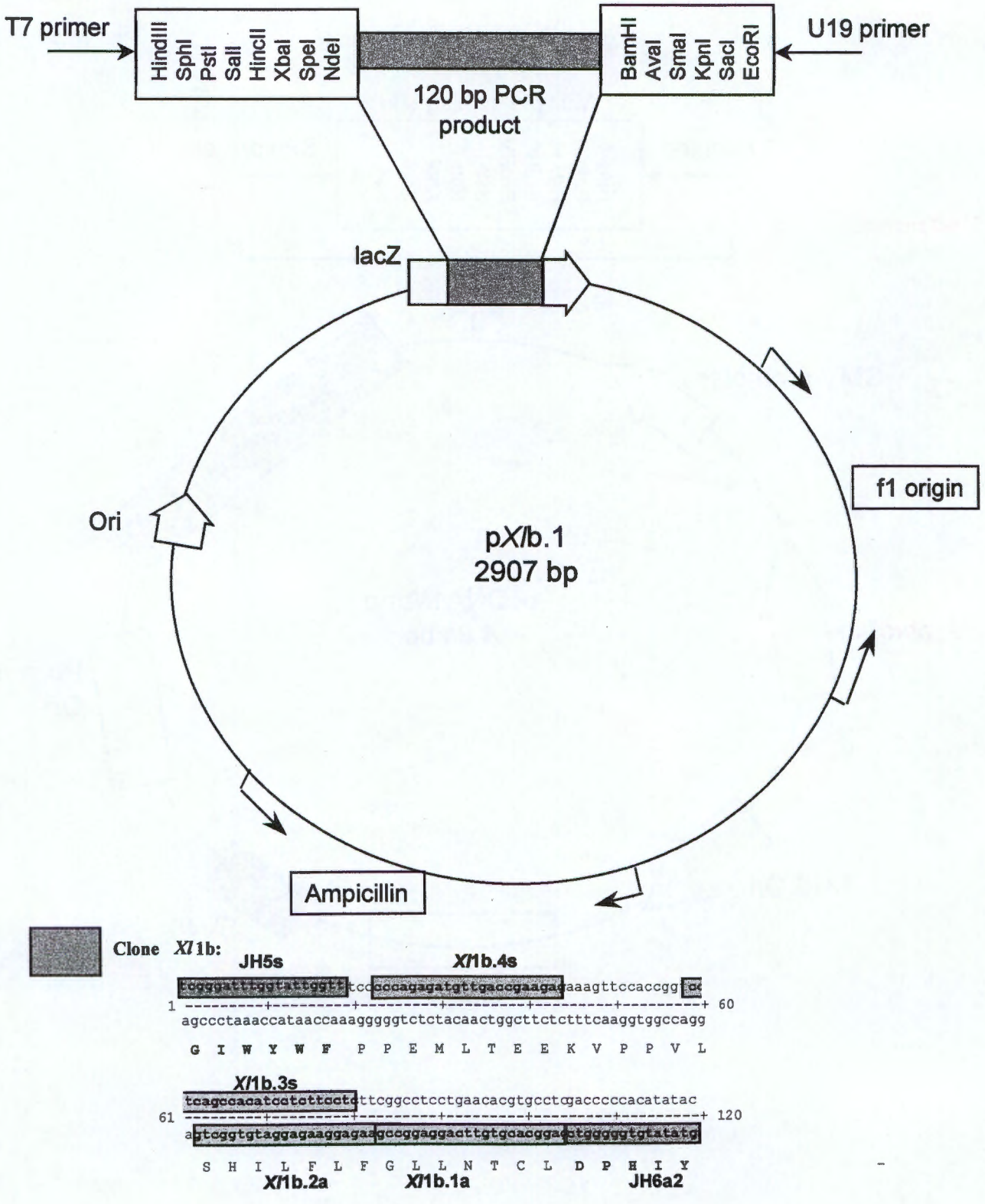
```
    atggaaggtccaacatctactgctgtcaccatgcaatcagtcttctgaaaggcaaggcat
361 ---------+---------+---------+---------+---------+---------+ 420
    taccttccaggttgtagatgacgacagtggtacgttagtcagaagactttccgttccgta
    M  E  G  P  T  S  T  A  V  T  M  Q  S  V  F  *  K  A  R  H  -

    tggattttaattcattttgatctacatatcgacctagcatgcccatatatatacttttc
421 ---------+---------+---------+---------+---------+---------+ 480
    acctaaaattaagtaaaactagatgtatagctggatcgtacgggtatatatatgaaaaag
    W  I  L  I  H  F  D  L  H  I  D  L  A  C  P  Y  I  Y  F  F  -
```

*X*l1a.1a

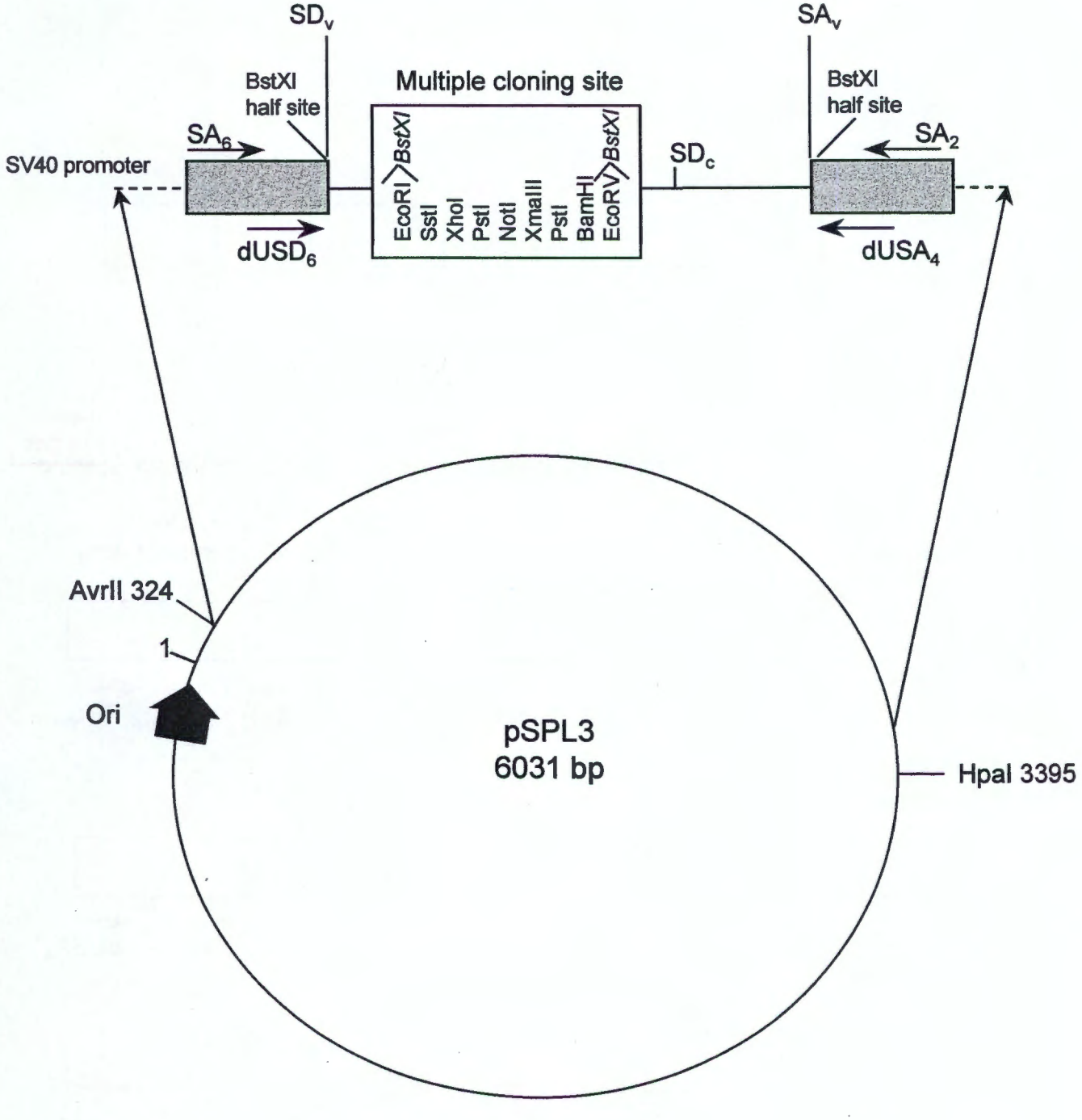
Appendix 7.6: Map of pcDNA1/Amp expression vector, showing the multiple cloning site



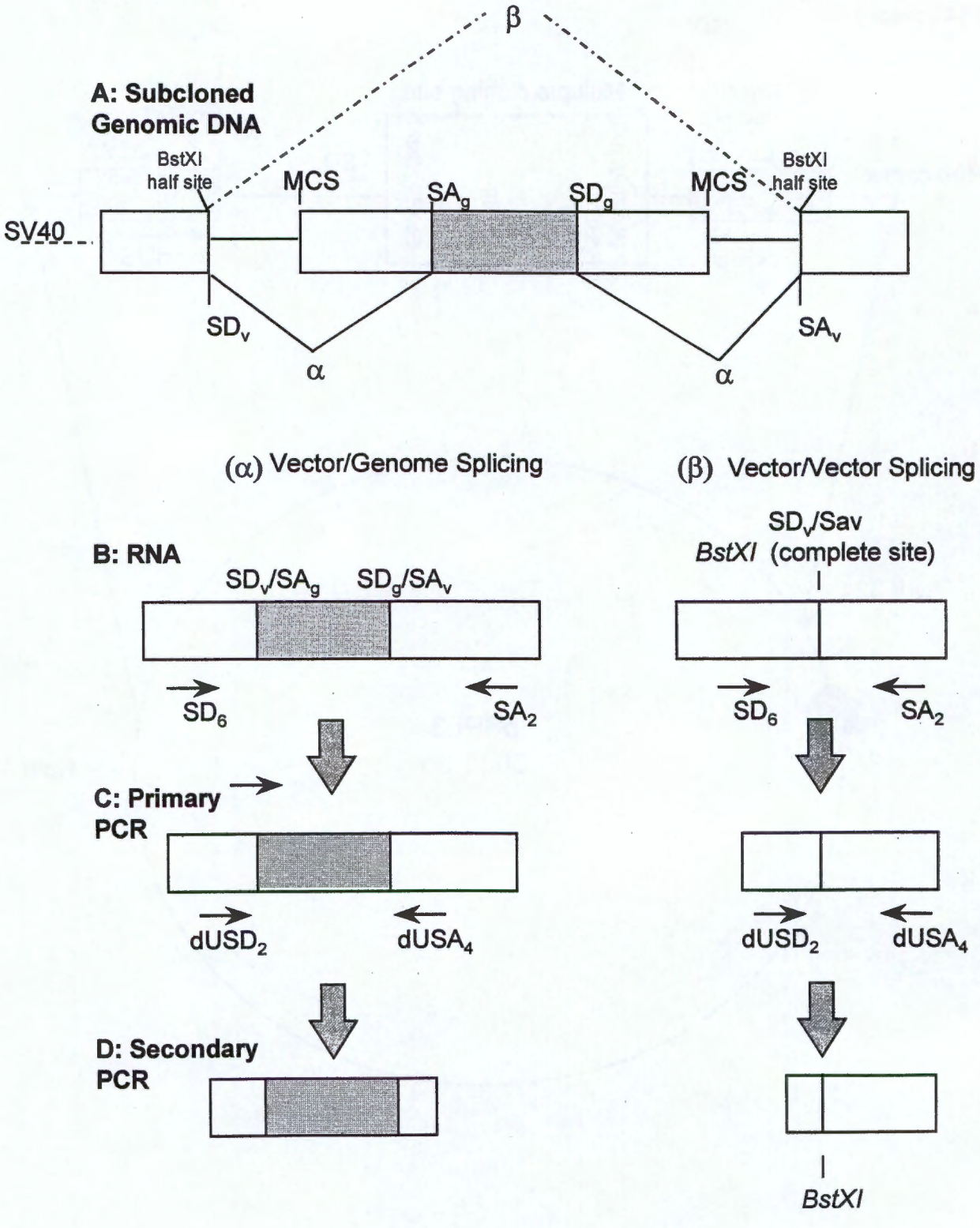
Appendix 7.7: Map of p*Xl*b.1 showing the nucleotide sequence and the translated amino acid sequence of the insert. Primers used for PCR amlification of cDNA are also shown.



Appendix 7.8a: Map of pSPL3 exon trapping vector. The upper region shows the HIV-1 *tat* sequences. Shaded blocks represent exon sequences, where SD_v = vector splice donor and SA_v = vector splice acceptor. solid line represents the intron sequence, where SD_c = cryptic splice donor in the intron region. PCR primers are indicated by arrows. Diagram is not drawn to scale.



Appendix 7.8b: Potential splicing events in the exon trapping procedure. A; represents the genomic fragment cloned into the pSPL3 vector. B; RNA extracted for transfected COS-1 cells, is converted to cDNA and amplified in C. This amplified DNA is reamplified in D. SD_g = genomic DNA splice donor, SA_g = genomic DNA splice acceptor, SD_v = vector splice donor, SA_v = vector splice acceptor.



Appendix 7.9: Nucleotide and deduced amino acid sequence of pXb from 5' and 3' Marathon (Clonetech) PCR

```
(Linear) MAP of: Frog2n.;1  check: 2096  from: 1  to: 404

        ggagaccatctataacatgttcaccttcttctgcctcttcctgctgcgtctcctcatcat
      1 ---------+---------+---------+---------+---------+---------+ 60
        cctctggtagatattgtacaagtggaagaagacggagaaggacgacgcagaggagtagta

b         E  T  I  Y  N  M  F  T  F  F  C  L  F  L  L  R  L  L  I  M  -

        ggtgtcctgctatacacgcatcctgatggagatatcgcacaagatgaaggcaacttgtgt
     61 ---------+---------+---------+---------+---------+---------+ 120
        ccacaggacgatatgtgcgtaggactacctctatagcgtgttctacttccgttgaacaca

b         V  S  C  Y  T  R  I  L  M  E  I  S  H  K  M  K  A  T  C  V  -

        ttcctccaaagaaattgacctccggcgctcatcaaataacatcccccgggctcgaatgag
    121 ---------+---------+---------+---------+---------+---------+ 180
        aaggaggtttctttaactggaggccgcgagtagtttattgtagggggcccgagcttactc

b         S  S  K  E  I  D  L  R  R  S  S  N  N  I  P  R  A  R  M  R  -

        aacattgaaaatgtccttggtgatagtgctgaccttcatcgtgtgctggactccatatta
    181 ---------+---------+---------+---------+---------+---------+ 240
        ttgtaacttttacaggaaccactatcacgactggaagtagcacacgacctgaggtataat

b         T  L  K  M  S  L  V  I  V  L  T  F  I  V  C  W  T  P  Y  Y  -

        tctcctagggatatggtactggtttcccccagagatgttgaccgaagagaaagttccacc
    241 ---------+---------+---------+---------+---------+---------+ 300
        agaggatccctataccatgaccaaaggggtctctacaactggcttctctttcaaggtgg

b         L  L  G  I  W  Y  W  F  P  P  E  M  L  T  E  E  K  V  P  P  -

        ggtcctcagccacatcctcttcctcttcggcctcctgaacacgtgcctcgacccccaaat
    301 ---------+---------+---------+---------+---------+---------+ 360
        ccaggagtcggtgtaggagaaggagaagccggaggacttgtgcacggagctgggggttta

b         V  L  S  H  I  L  F  L  F  G  L  L  N  T  C  L  D  P  Q  I  -

        atacggctcgagtgtaagccgaattctgcagatatccatcacac
    361 ---------+---------+---------+---------+---- 404
        tatgccgagctcacattcggcttaagacgtctataggtagtgtg

b         Y  G  S  S  V  S  R  I  L  Q  I  S  I  T    -
```

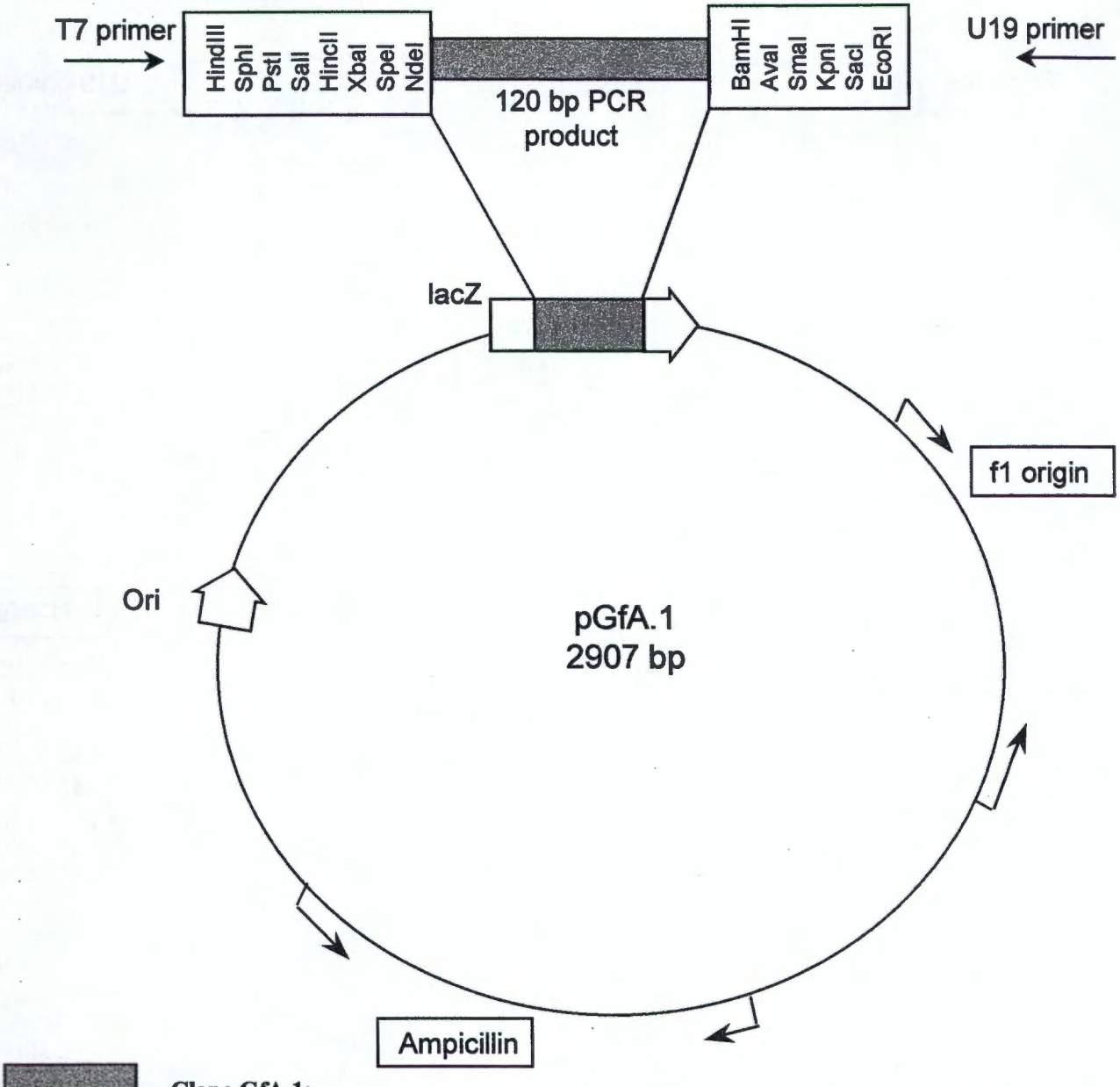
Appendix 7.10: Nucleotide sequence of the exon trapping subclone aligned to the *X. laevis* 28 S, 18 S and 5.8 S ribosomal RNAs, to which it showed a 96.4% identity

ET9: Exon trapping subclone number 9
Accession number of the ribosomal RNA to which ET9 aligns: Gb_Ov:Xlrn01

LOCUS XLRN01 8153 bp DNA VRT 28-FEB-1992
DEFINITION *Xenopus laevis* genes for 18S, 5.8S and 28S ribosomal RNAs.
ACCESSION X02995 J00999 J01001 J01002 J01003 J01006 J01007 K01369 K01370 K01371 K01372 K01373 K01376 K01535 V01444 V01445 V01446 V01447 V01448 V01449 V01450 V01451 V01452 V01453 V01454 V01456 X00136 X01071 . . .
SCORES 96.4% identity in 140 bp overlap

```
          10         20         30         40         50         60
Et9       CACTATAGGGCTCGAGCGGCCGCCCGGGCAGGTCCTTTGATCGCTCCATCTGTTACTTGG
                                          | ||||||||||||||||||||||||||||
Xlrn01    AACTGCGAATGGCTCATTAAATCAGTTATGGTTCCTTTGATCGCTCCATCTGTTACTTGG
          1120       1130       1140       1150       1160       1170

          70         80         90        100        110        120
Et9       ATAACTGTGGTAATTCTAGAGCTAATACATGCCGACGAGCGCTGACCCCCAGGGATGCGT
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Xlrn01    ATAACTGTGGTAATTCTAGAGCTAATACATGCCGACGAGCGCTGACCCCCAGGGATGCGT
          1180       1190       1200       1210       1220       1230

        130        140        150        160        170
Et9       GCATTTATCAGACCAAAACCAATCCGGGG-CCCCGCG-CCCGCTCGCTTT
          |||||||||||||||||||||||||||| ||||||| |||| ||||||
Xlrn01    GCATTTATCAGACCAAAACCAATCCGGGGCCCCCGCGCCCCGGCCGCTTTGGTGACTCTA
          1240       1250       1260       1270       1280       1290
```

Appendix 7.11: Map of pGfA.1 showing the nucleotide and translated amino acid sequences of the insert.



Clone GfA.1:

GfA.2s GfA.4s

ggaatatggtactggttccagccggagatgctgaaggtcacgcccgagtacatccatcac

1 ---------+---------+---------+---------+---------+---------+ 60

cctgataccatgaccaaggtcggcctctacgacttccagtgcgggctcatgtaggtagtg

G I W Y W F Q P E M L K V T P E Y I H H -

-..... JH 5s GfA.3as

ctgctcttcgtgttcgggaacctgaacacctgctgtgatccccttatctac

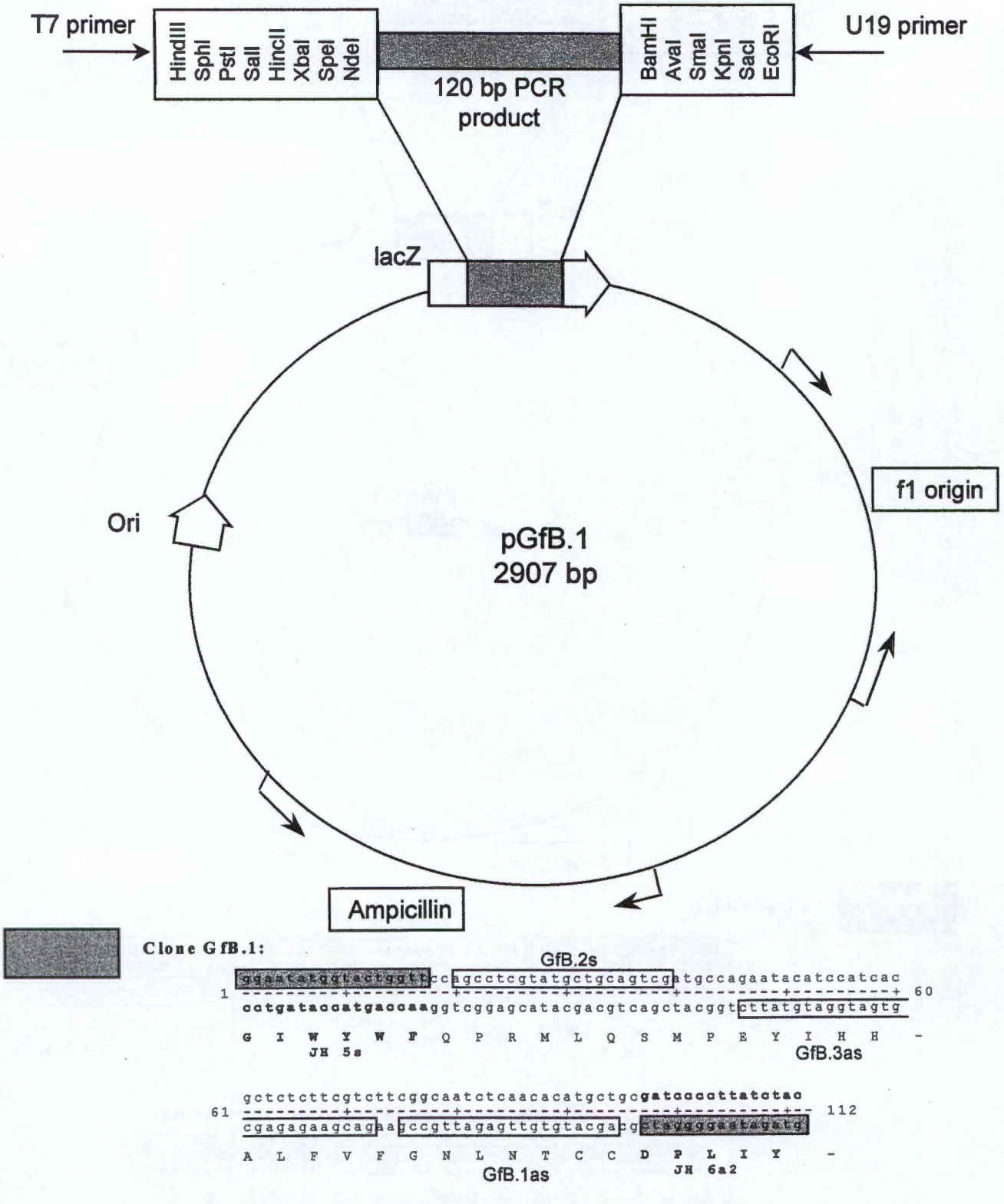
61 ---------+---------+---------+---------+---------+-- 112

gacgagaagcacaagcccttggacttgtggacgacactagggaatagatg

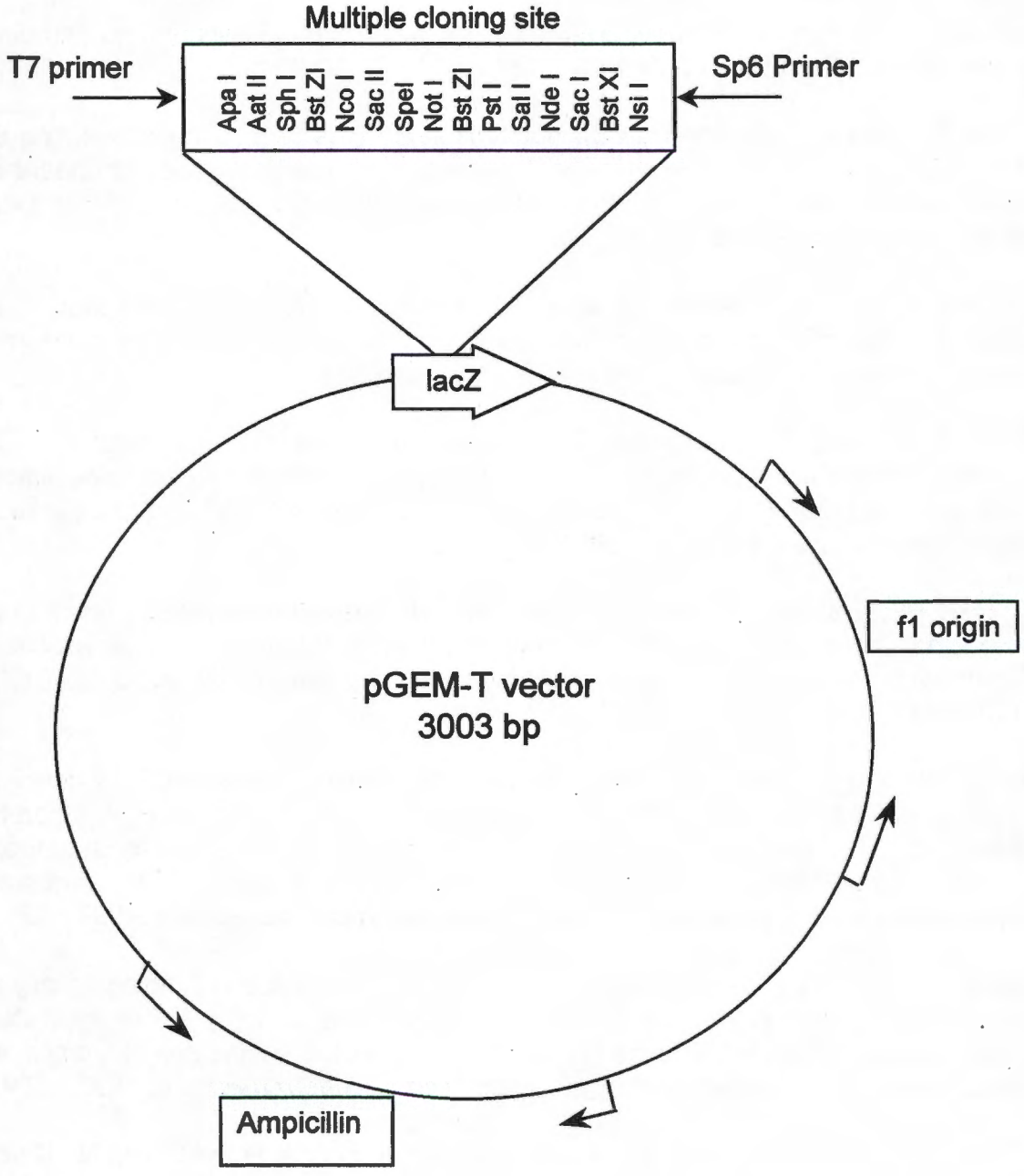
L L F V F G N L N T C C D P L I Y -

GfA.1as JH 6a2

Appendix 7.12: Map of pGfB.1 showing the nucleotide and translated amino acid sequences of the insert.



Appendix 7.13: Map of pGEM-T PCR cloning vector (Promega)



Appendix 7.14.

Publications

Troskie BE., Ott TR., Fromme B, Hapgood JP., Millar RP., and Illing N. Cloning and chimeric exchange of extracellular domains of the *Xenopus laevis* and human GnRH receptors reveals determinants for agonist binding. *In preparation*

Millar R., Conklin D., Lofton-Day C., Hutchinson E., Troskie B., Illing N., Sealfon SC., and Hapgood J. A novel human GnRH receptor homologue gene: Abundant and wide tissue distribution of the antisense transcript. *Submitted Molecular Endocrinology, July 1998*

Troskie B., Illing N., Rumbak E., Sun Y-M., Sealfon S., Conklin D., Hapgood J., and Millar R. Identification of three putative GnRH receptor subtypes in vertebrates. *Submitted Gen Comp Endo, May 1998*

Illing N., Troskie B., Nahorniak C., Hapgood J., Peter R., and Millar R. Two gonadotropin-releasing hormone receptor subtypes with distinct ligand selectivity and differential distribution in brain and pituitary cloned from goldfish (*Carassius auratus*). *Submitted Proc Natl Acad Sci 1998*

Troskie BE., Hapgood J., Millar RP., and Illing N. The *Xenopus laevis* gonadotropin-releasing hormone receeptor: cloning and characterisation. *Advances in Comparative Endocrinology. In Kawashima and Kikuyama (Eds) Monduzzi Editore, 1997 p747.*

Millar RP.,Troskie BE., Sun Y-M., Ott TR., Wakefield I., Myburgh D., Pawson A., Davidson JS., Flanagan C., Katz A., Hapgood J., Illing N., Weinstein H., Sealfon SC., Peter RE., Terasawa E., and King JA. Plasticity in th structural and functional evolution of GnRH: a peptide for all seasons. *Advances in Comparative Endocrinology. In Kawashima and Kikuyama (Eds) Monduzzi Editore, 1997 p13.*

Millar RP.,Troskie BE., and Flanagan C. Comparative receptor binding affinity and inositol phosphate production potency of D-Leu^6 and D-Trp^6 GnRH agonists on COS-1 cells transfected with the human GnRH receptor. *Advances in Comparative Endocrinology. In Kawashima and Kikuyama (Eds) Monduzzi Editore, 1997 p559.*

Troskie BE., King JA., Millar RP., Peng Y., Kim J., Figueras H., and Illing N. Chicken GnRH II-like peptides and a GnRH Receptor selective for Chicken GnRH II in Amphibian Sympathetic Ganglia. *Neuroendocrinology 1997;65,6:396-402*